Aus dem Fachbereich Medizin
der Johann Wolfgang Goethe-Universität Frankfurt am Main

Gustav-Embden Zentrum für Biologische Chemie
Institut für Biochemie I – Pathobiochemie
Direktor: Prof. Dr. Bernhard Brüne

Regulation of arginase II expression in macrophages by supernatants of apoptotic cells

Dissertation

zur Erlangung des Doktorgrades der
theoretischen Medizin des Fachbereichs Medizin
der Johann Wolfgang Goethe-Universität
Frankfurt am Main

vorgelegt von
Vera Diana Barra
aus Coimbra (Portugal)

Frankfurt am Main, 2010

Dekan:	Prof. Dr. med. J. Pfeilschifter
Referent:	Prof. Dr. rer. nat. B. Brüne
Koreferentin:	Prof. Dr. med. D. Meyer zu Heringdorf

Tag der mündlichen Prüfung: 11.03.2011

Das tiefste und erhabenste Gefühl, dessen wir fähig sind,
ist das Erlebnis des Mystischen.
Aus ihm allein keimt wahre Wissenschaft.
Wem dieses Gefühl fremd ist,
wer sich nicht mehr wundern und in Ehrfurcht verlieren kann,
der ist seelisch bereits tot.

Albert Einstein

deutsch-amerikanischer Physiker (1879 - 1955)

Index

List of Figures

List of Tables

Abbreviations

AA	Arachidonic acid
ABC	ATP-binding cassette transporter
ACs	Apoptotic cells
ACAMPs	Apoptotic cell-associated molecular patterns
ADP	Adenosine diphosphate
AKAP	A kinase-anchoring protein
APC	Antigen presenting cell
APS	Ammonium persulfate
ARG	Arginase
ATP	Adenosine triphosphate
β_2-GPI	β_2-glycoprotein I
bp	Base pairs
BSA	Bovine serum albumin
C1P	Ceramide-1-phosphate
cAMP	Cyclic adenosine monophosphate
C/EBP	CCAAT/enhancer binding protein
CDase	Ceramidase
CM	AC-conditioned medium
CSF	Colony-stimulating factor
COX	Cyclooxygenase
CREB	cAMP responsive element binding protein
C1q	Complement fragment 1
dH_2O	Distilled water
ddH_2O	Double-distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide
DTT	Dithiothreitole
EDTA	Ethylene diamine tetra acetate
EGF	Epidermal growth factor
EGFP	Enhance green fluorescent protein
EGTA	Ethylene glycol tetra acetate
EIA	Enzyme immunoassay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
EP	E prostanoid receptor
EPAC	Exchange protein directly activated by cAMP
FACS	Fluorescence-activated cell sorting
FcγR	Fcγ receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor

GTP	Guanosine triphosphate
IBMX	3-isobutyl-1-methylxanthine
ICAM3	Intercellular adhesion molecule 3
iC3b	Complement fragment inactivated C3b
IFN	Interferon
IL	Interleukin
IG	Immunoglobulin
IP_3	Inositol 1,4,5-trisphophate
IRF	Interferon regulatory factor
iNOS	Inducible NO-Synthase
$iPLA_2$	Cytosolic calcium-independent phospholipase A_2
JNK	C-Jun N-terminal kinase/stress-activated protein kinase
KCl	Potassium chloride
KH_2PO_4	Potassium hydrogen phosphate
$KHCO_3$	Potassium hydrogen carbonate
LDL	Low density lipoprotein
LOX	oxLDL receptor
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPS	Lipopolysacharide
LXR	Liver X receptor
mAKAP	Muscle-specific A kinase-anchoring protein
MAPK	Mitogen-activated protein kinases
MEK	Mitogen-activated protein kinase kinase
Mer	Mer tyrosine kinase receptor
MΦs	Macrophages
MFG-E8	Milk-fat globule epidermal growth factor 8
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
$MgSO_4$ x 7 H_2O	Magnesium sulfate 7-hydrate
MHC	Major histocompatibility complex
mpARG II	Murine arginase II promoter
MR	Mannose receptor
mRNA	messenger ribonucleic acid
mod.CD31	Modified CD31
Na_2HPO_4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NaF	Sodium fluoride
NCs	Necrotic cells
NCoR	Nuclear receptor co-repressor
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
NH_4Cl	Ammonium chloride
NES	Nuclear export signal

NO	Nitric oxide
oxLDL	oxidized lipoproteins
PAF	Platelet-activating factor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PPARγ	Peroxisome proliferator-activated receptor γ
PdtCho	Phosphatidylcholin
PtdSer	Phosphatidylserine
PtdSerR	Phosphatidylserine receptor
PCR	Polymerase chain reaction
PG	Prostaglandin
PI3K	Phosphatidylinositol-3 kinase
PK	Protein kinase
PL	Phospholipase
PMSF	Phenylmethylsulphonylfluoride
PtdSer	Phosphatidylserine
qPCR	quantitative Polymerase chain reaction
R	Arginine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI	Roswell park memorial institute
RSK	Ribosomal S6 kinase
RT	Room temperature
S1P	Sphingosine-1-phosphate
$S1P_{1-5}$	S1P receptor 1-5
SDS	Sodium dodecyl sulfate
SE	Standard error
SK	Sphingosine kinase
SMase	Sphingomyelinase
SRA	Scavenger receptor A
TAM	Tumor-associated macrophages
TEMED	Tetraethylendiamine
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	12-Tetradecanoyl-phorbol-13-acetate
Tulp	Tubby-like protein
VEGF	Vascular endothelial growth factor
VnR	Vitronectin receptor

1 Summary

Apoptotic cell (AC)-derived factors alter the physiology of macrophages (MΦs) towards a regulatory phenotype that is characterized by enhanced production of anti-inflammatory mediators, an attenuated pro-inflammatory cytokine profile and reduced nitric oxide (NO) formation. Impaired NO production in response to ACs or AC-conditioned medium (CM) is facilitated by arginase II (ARG II) expression, which competes with inducible NO synthase for L-arginine.

In this study, I investigated the signaling pathway that allowed CM to up-regulate ARG II in MΦs. A sphingolipid, further identified as sphingosine-1-phosphate (S1P), was required but authentic S1P alone only produced small effects. S1P acted synergistically with a so far unidentified factor to elicit high ARG II expression. S1P signaled through S1P receptor 2 ($S1P_2$), since the $S1P_2$-antagonist JTE013 and siRNA knock-down of $S1P_2$ prevented ARG II up-regulation. Further, inhibition and knock-down of extracellular signal-regulated kinase 5 (ERK5) attenuated CM-mediated ARG II protein induction. Exploring ERK5-dependent transcriptional regulation, promoter deletion and luciferase reporter analysis of the murine ARG II promoter (mpARG II) suggested the involvement of cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB). This was confirmed by EMSA analysis and decoy-oligonucleotides scavenging CREB, thereby preventing it from activating target genes and thus, blocking ARG II expression. I concluded that AC-derived S1P binds to $S1P_2$ and acts synergistically with other factors to activate ERK5 and concomitantly CREB. This signaling cascade shapes an anti-inflammatory MΦ phenotype by ARG II induction.

Further investigations of ERK5-dependent CREB activation suggested an indirect mechanism implying that ERK5 inhibited phosphodiesterase 4 (PDE4) and thus, prevented hydrolysis of cAMP. Since S1P-dependent ERK5 activation presumably inhibited PDE4, subsequent cAMP accumulation led to enhanced PKA activity and CREB-mediated transcription. The unidentified factor(s) besides S1P probably provoked the required elevation of cAMP production in MΦs. Indeed, pharmacological inhibition of cAMP-producing adenylyl cyclase with SQ22536 as well as cAMP-dependent protein kinase A (PKA) with KT5720 suggested cAMP to be involved in CM-mediated ARG II up-regulation.

Furthermore, forskolin-dependent activation of adenyly cyclase and simultaneous rolipram-mediated inhibition of PDE4 mimicked CM-induced ARG II expression.

Considering these findings, I propose that one or several unidentified factors in CM provoke cAMP production in MΦs. In parallel, AC-derived S1P activates ERK5, which inhibits PDE4-dependent cAMP hydrolysis, further raising intracellular cAMP levels. Thus, unrestricted continuous cAMP signaling via PKA/CREB, results in a time-dependent and sustained ARG II induction.

2 Zusammenfassung

Apoptotische Zellen (ACs) setzen Faktoren frei, die die biologischen Reaktionen von Makrophagen (MΦs) beeinflussen, indem sie einen regulatorischen MΦ-Phänotyp induzieren. Dieser ist sowohl durch eine erhöhte anti- als auch eine verminderte pro-inflammatorische Botenstoffproduktion, sowie durch eine reduzierte Stickstoffmonoxid(NO)-Synthese gekennzeichnet. Die Reduktion der NO-Produktion durch ACs oder AC-konditioniertes Medium (CM), erfolgt durch Induktion der Arginase II (ARG II), die mit der NO-Synthase um das Substrat L-Arginin konkurriert.

In der vorliegenden Arbeit untersuchte ich die CM-induzierten, zellulären Signalwege, die zur ARG II Expression in MΦs führen. Das im CM vorhandene Sphingolipid Sphingosin-1-Phosphat (S1P) war erforderlich für die ARG II Induktion. Da authentisches S1P alleine nur geringe Effekte zeigte, wirkte es höchstwahrscheinlich synergistisch mit einem oder mehreren, bis jetzt unidentifizierten, Faktoren um eine starke ARG II Expression auszulösen. Dabei aktivierte S1P den S1P Rezeptor 2 ($S1P_2$), da der $S1P_2$-Antagonist JTE013 und die Inhibition der Genexpression durch siRNA die ARG II Induktion verhinderten. Weiter konnte die CM-bedingte ARG II Proteininduktion durch Hemmung und siRNA Knockdown der ‚extracellular signal-regulated kinase 5' (ERK5) reduziert werden. Die Untersuchung der ERK5-vermittelten Transkriptionsregulation durch Luziferase-Reporteranalysen unter Verwendung von Deletions-Konstrukten des murinen ARG II Promotors (mpARG II) deutete auf die Beteiligung des ‚cyclic adenosine monophosphate (cAMP) responsive element binding protein' (CREB) an der ARG II Regulation hin. Dies wurde durch EMSA-Analysen und den Einsatz von Oligonukleotiden, die spezifisch CREB binden, bestätigt. Hier kann das an Oligonukleotide gebundene CREB seine Zielgene nicht aktivieren und dementsprechend auch nicht die Transkription der ARG II induzieren. Daraus schließe ich, dass S1P in CM zur ARG II Induktion in MΦs beiträgt, indem es an $S1P_2$ bindet und synergistisch mit anderen Faktoren zur Aktivierung von ERK5 und anschließend von CREB führt. Diese Signalkaskade induziert einen anti-inflammatorischen MΦ Phänotyp, indem sie ARG II hochreguliert.

Die weitere Untersuchung der ERK5-vermittelten CREB Aktivierung ließ einen indirekten Mechanismus vermuten, bei dem ERK5 die Phosphodiesterase 4 (PDE4) hemmt und damit den Abbau von cAMP verhindert. Da die S1P-abhängige ERK5 Aktivierung PDE4 vermutlich inhibiert, steht der PKA mehr cAMP zur Verfügung, um CREB zu aktivieren. Der/die unidentifizierte(n) Faktor(en) neben S1P bewirkte(n) wahrscheinlich die benötigte cAMP-Synthese in MΦs. In der Tat verringerte die pharmakologische Hemmung der cAMP-produzierenden Adenylatzyklase mit SQ22536, sowie der cAMP-aktivierten Proteinkinase A (PKA) mit KT5720, die CM-vermittelte ARG II Induktion. Außerdem imitierten die Aktivierung der Adenylatzyklase durch Forskolin und die gleichzeitige Hemmung der PDE4 durch Rolipram die CM-bedingte ARG II Induktion.

Angesichts dieser Befunde schlage ich vor, dass ein oder mehrere unidentifizierte Faktoren im CM zur cAMP-Produktion in MΦs führen. Parallel dazu aktiviert S1P aus ACs ERK5, wodurch PDE4 inhibiert und damit die cAMP-Hydrolyse gehemmt wird. Auf diese Weise bewirkt das anhaltende cAMP-Signal die PKA-vermittelte CREB Aktivierung, die die zeitabhängige und nachhaltige ARG II Expression verursacht.

3 Introduction

3.1 Homeostasis and apoptotic cell death

3.1.1 Tissue homeostasis

The concept of homeostasis was developed by Walter Bradford Cannon and popularized in its book *The Wisdom of the Body* published in 1932. Homeostasis (Greek: *hómos* = similar, *histemi* = standing still) is defined as the property of an organism to regulate internal environments and maintain a stable, constant condition by adjusting its physiological processes. In adult organisms, the number of cells is kept relatively constant. The control mechanism ensuring this tissue homeostasis is achieved by fine-tuning of cellular signaling, resulting in a balanced rate of mitosis and cell death. Dysregulation of these signaling pathways may cause serious disorders, like the development of cancer in case cells divide faster than they die, or cell loss leading to organ dysfunction if cells divide slower than they undergo apoptotic cell death.

3.1.2 Apoptosis

The process of cell death was first discovered in 1842 by Carl Vogt and more precisely described in 1885 by Walther Flemming, but it was not until 1965 that John Foxton Ross Kerr was able to discriminate between programmed cell death called apoptosis (Greek: *apo* = from, *ptosis* = falling) and traumatic cell death or necrosis (Greek: *nékros* = dead) using electron microscopy (Kerr, 1965). The term apoptosis, used in Greek for the ‘dropping off’ of leaves from trees, was proposed by James Cormack, professor of Greek language (University of Aberdeen, Scottland), in 1972 to describe the natural process of cell death in multicellular organisms. The function of apoptosis consists in removing old, damaged or infected cells to spare the surrounding tissue from detrimental cellular constituents that would be released in case of necrosis, thus contributing to tissue homeostasis.

Apoptosis involves biochemical events leading to morphological changes of cells including cell shrinkage, loss of membrane asymmetry, blebbing, chromatin condensation, DNA and nuclear fragmentation. Cell disintegration

occurs by formation of so-called ‘apoptotic bodies’ with intact membrane integrity (Kerr et al., 1972; Wyllie et al., 1980). The whole process of apoptosis is tightly regulated, energy-dependent and immunologically ‘silent’ when terminated properly through clearance of apoptotic bodies by professional phagocytes. In contrast, necrosis caused by traumata, such as toxins, ischemia, mechanical stress or heat, provokes cell swelling and membrane rupture. As a consequence, cellular constituents are released into the surrounding tissue subsequently causing inflammation (Savill et al., 2002; Savill and Fadok, 2000) (FIGURE 3.1).

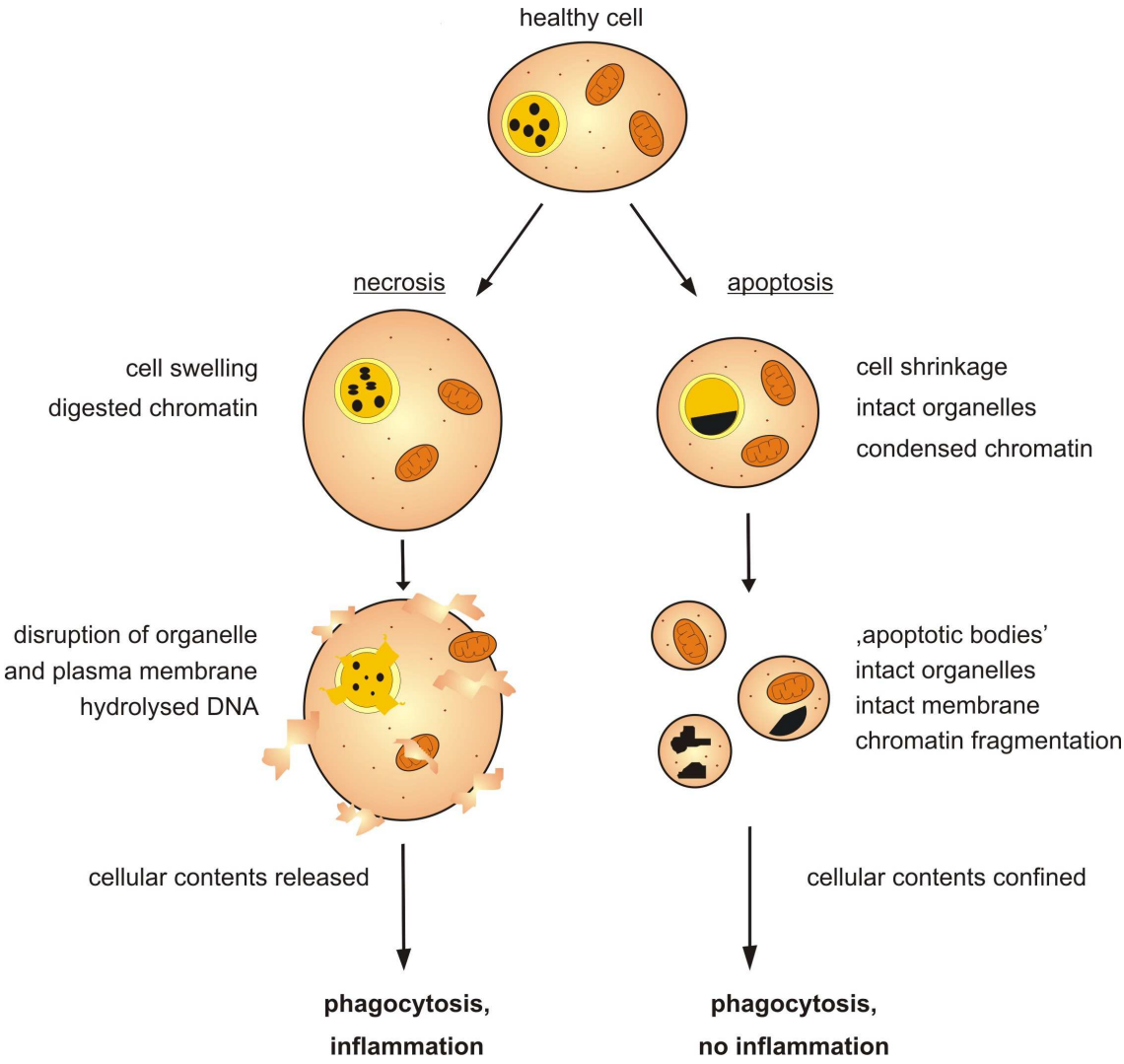

FIGURE 3.1: Scheme of apoptosis vs. necrosis.
During apoptosis, cells are degraded to ‘apoptotic bodies’ and removed without provoking inflammation. Necrotic cells on the other hand loose their membrane integrety, thereby releasing cellular constituents and causing inflammation.

When the phagocytic capacity is over-saturated *in vivo*, apoptotic cells (ACs) undergo ‘secondary necrosis’, which provokes inflammation and might induce autoimmunity (Albert, 2004; Savill et al., 2002).

3.2 Phagocytosis of apoptotic cells

In order to prevent secondary necrosis and the implicated inflammatory consequences, ACs have to be removed quickly. This termination of the apoptotic program is achieved by professional phagocytes, e.g. macrophages (MΦs) and can be subdivided into three steps. First, phagocytes are attracted to the site of apoptosis, then they specifically recognize ACs, and finally they engulf ACs and their ‘apoptotic bodies’.

3.2.1 Phagocyte attraction

As phagocytes may not be in close proximity to ACs, they have to obtain molecular signals directing them correctly. The mediators of such molecular signals are called chemoattractants and some have been identified and reported to be secreted by ACs. For example, apoptotic fibroblast-derived thrombospondin 1 (TSP1) was shown to recruit MΦs and, as a heterodimer with CD36 on the fibroblast, further to signal for recognition of ACs (Moodley et al., 2003). Furthermore, calcium-independent phospholipase A_2 ($iPLA_2$) is activated during apoptosis and hydrolyzes the membrane glycerophospholipid phosphatidylcholine thereby releasing lysophosphatidylcholine (LPC), which induces monocyte and MΦ migration *in vitro* and *in vivo* (Lauber et al., 2003) probably by binding to the G protein coupled receptor G2A (Peter et al., 2008). In addition to glycerophospholipid metabolism, sphingolipid metabolism is also active during apoptosis, producing among others sphingosine-1-phosphate (S1P) (Weigert et al., 2006), which has chemoattractant properties towards monocytes and MΦs *in vitro* (Gude et al., 2008). ACs probably also recruit leukocytes by mimicking the action of IL-8, which is known as a potent cytokine mediating leukocyte infiltration into inflamed tissues (Baggiolini et al., 1989). Furthermore, IL-8 has also been reported to contribute to monocyte recruitment *in vitro* by triggering firm binding of monocytes to an endothelial monolayer expressing E-selectin (Gerszten et al., 1999). Indeed, during apoptosis, cells release the endothelial monocyte-activating polypeptide II (EMAP II) by cleaving

aminoacyl-tRNA synthetase, an enzyme crucially involved in protein translation. EMAP II is able to mimic IL-8 by binding to the IL-8 receptor on MΦs (Wakasugi and Schimmel, 1999). However, ACs also secrete chemorepellants like lactoferrin, a potent inhibitor of granulocyte migration *in vitro* and *in vivo* (Bournazou et al., 2009). Thus, ACs tightly regulate inflammatory processes by recruiting phagocytes and ensuring their clearance on the one hand, and by preventing the attraction of additional inflammatory cells on the other.

3.2.2 Recognition and removal of apoptotic cells

The recognition and phagocytosis of ACs *in vivo* is achieved by a multiplicity of membrane receptors. Since the identification of the first MΦ receptor (vitronectin receptor (VnR)($\alpha_v\beta_3$-integrin)) mediating up-take of ACs (Savill et al., 1990), further *in vitro* studies indicate that phagocytosis of ACs is an extremely complex process. Many phagocyte receptors, different bridging molecules as well as several surface markers of ACs called apoptotic cell-associated molecular patterns (ACAMPs) are involved in this procedure. The complexity of this process probably facilitates specific recognition, improved binding and an increased phagocytic potential. The involvement of the different receptors in the termination of apoptosis is discussed in the following sections.

3.2.2.1 Innate recognition of 'nonself' molecules by phagocytes

Some phagocyte receptors that mediate AC up-take are elements of the innate immune system, like the lipopolysaccharid (LPS) receptor CD14 (Devitt et al., 1998), the complement fragment 1 (C1q) (Taylor et al., 2000) and proteins of the collectin family (Ogden et al., 2001; Schagat et al., 2001) (see FIGURE 3.2 A). During innate immunity, they initiate inflammatory and immune reactions in response to pathogens (Savill et al., 2002). 'Eat me' signals on the surface of ACs were denominated ACAMPs in accordance with the designation for recognition molecules on the surface of pathogens, so-called pathogen-associated molecular patterns (PAMPs). Despite these similarities, unlike phagocytosis of pathogens, the up-take of ACs does not provoke a pro-inflammatory response, but induces an active anti-inflammatory one. This points to a mechanism, which in spite of being initiated by the same receptors, triggers different signaling cascades with opposite effects (Savill et al., 2002).

3.2.2.2 Recognition of modified 'self' molecules by phagocytes

Beside the receptors mentioned in 3.2.2.1, ACs are also recognized by receptors, which are described to be responsible for detection of damaged or modified 'self' molecules. Examples for such receptors are the class-A scavenger receptor (SRA), the class-B scavenger receptor CD36, the lectin-like oxidized low-density lipoprotein receptor 1 (LOX1) and CD68, known to participate in endocytosis of oxidized low density lipoprotein (oxLDL). These receptors also recognize ACs and facilitate their clearance (Boss et al., 1998; Platt et al., 2000; Ren et al., 1995; Savill et al., 1992) (see FIGURE 3.2 B). Along this line, oxidation of surface molecules on ACs seems necessary for recognition/phagocytosis by MΦs (Chang et al., 1999; Kagan et al., 2003; Kagan et al., 2002). In this context, it was shown that monoclonal antibodies against oxLDL bind to ACs thereby preventing their phagocytosis (Chang et al., 1999). Beyond that, Kagan *et al.* showed that binding and activation of the Fas-receptor provoked reactive oxygen species (ROS) production in Jurkat cells and subsequent oxidized cytochrome c-dependent oxidation and presentation of phosphatidylserine (PtdSer) on the outer leaflet of the plasma membrane of ACs. The authors observed that phagocytosis of apoptotic Jurkat cells was inhibited by superoxide dismutase and catalase, which prevented oxidation of PtdSer while allowing it to remain externalized on these cells, suggesting oxydized PtdSer to be a prerequisite for recognition. Apart from some exceptions (Stowell et al., 2009), PtdSer is usually located in the inner leaflet of plasma membrane and this assymmetry is ensured by aminophospholipid translocase. During apoptosis, PtdSer is transported by a flip-flop mechanism to the outer leaflet of the plasma membrane, a process whose underlying mechanisms were not uncovered yet, but which seems to be based on inhibition of the translocase (Verhoven et al., 1995) and on Ca^{2+} and phosphorylation-dependent activation of a lipid scramblase (Frasch et al., 2000). However, PtdSer externalization during necrotic cell death has also been reported in some cells (Brouckaert et al., 2004), which then are engulfed by MΦs. Similar to ACs, such a clearance of NCs does not induce inflammation, thereby suggesting PtdSer as a general marker of different types of cell death sharing anti-inflammatory properties. Originally, Fadok *et al.* assumed to have identified a specific PtdSer receptor (PtdSerR) (Fadok et al., 2000). However, the role of

this presumed PtdSerR in phagocytosis has been refuted by some works (Bose et al., 2004; Cikala et al., 2004), one example being an investigation showing that PdtSerR-deficient cells were perfectly capable of recognizing, engulfing and responding to ACs (Mitchell et al., 2006). The authors concluded that PtdSerR is neither involved in specific innate recognition nor in engulfment of ACs.

Recent investigations identified Stabilin-2 (Park et al., 2008), Tim4 (Miyanishi et al., 2007) and BAI1 (Park et al., 2007) as novel direct PtdSer receptors (Bratton and Henson, 2008). Furthermore, PtdSer on ACs can be recognized and bound by bridge molecules like Gas6 and protein S (which bind to the tyrosine kinase receptor Mer) or milk fat globule epidermal growth factor 8 (MGF-E8) (recognized by VnR) (Lemke and Rothlin, 2008; Wu et al., 2006), as well as β_2-glycoprotein I (β_2-GPI), which attaches to the β_2-GPI receptor (Cocca et al., 2001) (see FIGURE 3.2 B). In addition, PtdSer on ACs seems to be able to activate components of the complement system (iC3b) thereby enhancing the up-take of ACs by MΦs (Mevorach et al., 1998). Beside the complement receptors, integrin and Fc receptors have also been suggested to take part in phagocytosis of ACs (Stuart and Ezekowitz, 2005). These observations point to a close relationship between innate recognition of foreign and modified self molecules.

Recently, Caberoy *et al.* identified tubby and tubby-like protein 1 (Tulp1) as new ‘eat me’ signals, which enhanced pagocytosis of retinal pigment cells by MΦs. Unlike Gas6, protein S or MGF-E8, these bridging molecules (recognized by Mer) lack PtdSer-binding activity, leading the authors to postulate other unidentified PtdSer-like molecules (Caberoy et al., 2009).

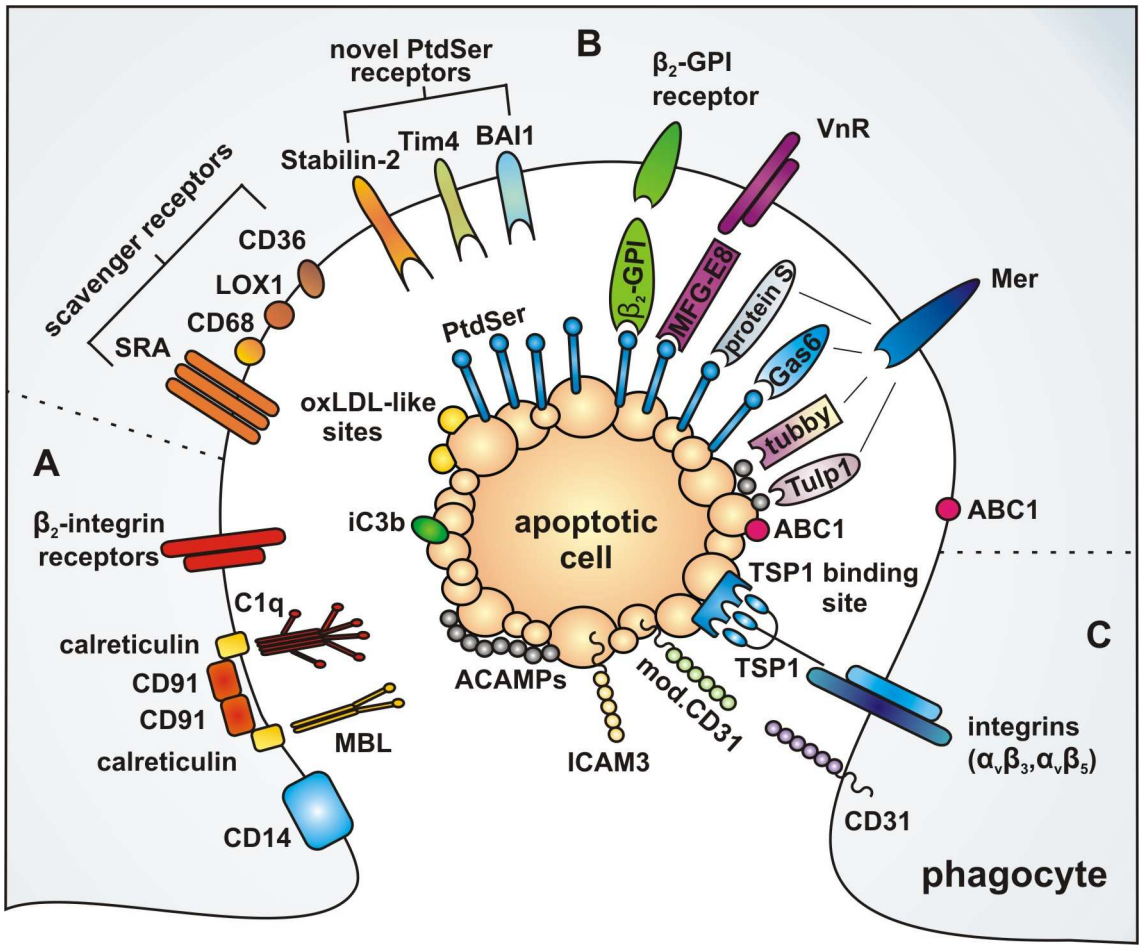

FIGURE 3.2: Mechanisms of apoptotic cell-recognition by macrophages.
A) The innate recognition of apoptotic cells (ACs) occurs via CD14, β_2-integrins binding the opsonic complement fragment inactivated C3b (iC3b) and CD91/calreticulin the C1q and the mannose-binding lectin (MBL). These receptors respectively their bridging molecules recognize and bind AC-associated molecular patterns (ACAMPs). **B)** Recognition of ACs as modified 'self' is mediated by several scavenger receptors, like class-A scavenger receptor (SRA), CD68, oxidized low-density lipoprotein (oxLDL) receptor 1 (LOX1) and CD36, which bind to oxidized membrane molecules since they have similar epitopes to oxLDL. Furthermore, presentation of PtdSer on the outer leaflet of the plasma membrane of ACs contributes to their recognition by macrophages (MΦs). PtdSer is bound either directly by PtdSer receptors, such as Stabilin-2, Tim4 and BAI1 or indirectly by bridging molecules and their respective receptors. The bridge molecule β_2-glycoprotein I (β_2-GPI) is recognized by the β_2-GPI receptor, milk fat globule epidermal growth factor 8 (MGF-E8) by the vitronectin receptor (VnR) and Gas6 and protein S by the tyrosine kinase receptor Mer. Other bridging molecules, like tubby and tubby-like protein 1 (Tulp1) bind to ACAMPs other than PtdSer and are also recognized by Mer. Redistribution of proteins in the plasma membrane of ACs as well as phagocytes by ATP-binding cassette transporter 1 (ABC1) may also facilitate this sort of recognition. **C)** Modified CD31 (mod.CD31) on ACs accounts for recognition of 'self' molecules that are unable to dislodge and disperse from phagocytes by strongly binding to the phagocytic CD31 and possibly facilitating binding of thrombospondin 1 (TSP1) to MΦ integrins. A further member of the immunoglobulin superfamily, the intercellular adhesion molecule 3 (ICAM3) might undergo similar modifications and thus also contribute to this sort of recognition.

3.2.2.3 Recognition of non-detaching 'self' molecules by phagocytes

The specific recognition of ACs also occurs by means of molecules, which normally promote adhesion and movement of living cells, like e.g. the intercellular adhesion molecule 3 (ICAM3) or TSP1 (Hughes et al., 1997; Savill et al., 1992). Thus, phagocytes need to discriminate between living, moving cells and ACs that have to be engulfed. However, the underlying mechanism remains elusive. A study of Brown *et al.* provides a possible explanation. The authors observed that apoptotic leukocytes, in contrast to living ones, cannot discern and process detachment signals from MΦs. The homophile connection of CD31 between phagocytes and living leukocytes provided repellent signals, thereby ensuring their active detachment. On the contrary the connection to dying leukocytes transformed the repulsive signals into attracting ones, thereby strengthening the bond between AC and phagocyte and promoting phagocytosis (Brown et al., 2002). Similar to these findings, the immunoglobulin ICAM3 might also undergo comparable modifications leading to the same effect (Savill et al., 2002) (see FIGURE 3.2 C). Thus, one could assume that the inability of ACs to dislodge and disperse from phagocytes is a general mechanism for the up-take of dying cells. Nonetheless, this connection may be unsufficient to induce phagocytosis, so that further signals, like e.g. the presentation of PtdSer on the outer leaflet of the plasma membrane, may be necessary (Hanayama et al., 2002).

3.2.2.4 Removal of apoptotic cells

Following recognition of ACs, phagocytes undergo cytoskeleton rearrangement in order to engulf them. The underlying mechanisms, linking AC-recognizing receptors of phagocytes and their actin filaments, are still unclear. A candidate is the CrkII-ELMO-DOCK180 complex in the plasma membrane, which mediates cytoskeleton changes by Rac1 activation and can be activated downstream of VnR or Mer (Akakura et al., 2004; Wu et al., 2006) thereby possibly linking PtdSer on ACs to phagocytosis. Another mechanism of Rac1 activation might occur via GULP, a downstream effector of CD91 that interacts with ATP-binding cassette transporter 1 (ABC1) on ACs (Lauber et al., 2004). Furthermore, the crucial role of transglutaminase type II (TG II) for the internalization, but not recognition and binding, of ACs was demonstrated by

TG II-ablation in mice, which resulted in defective clearance of ACs associated with the development of inflammation and auto-immunity (Falasca et al., 2005).
It is also worth mentioning that the processes of phagocytosis of ACs, necrotic cells (NCs) and pathogens are morphologically different. Electron microscopic investigations showed that ACs are phagocyted by a zipper-like mechanism, while pathogens are usually receptor-mediated endocytozed and NCs (as well as secondarily NCs) are macropinocytozed (Aderem and Underhill, 1999; Krysko et al., 2003). These differences reflect the complexity and strict regulation of the different phagocytic mechanisms and their effects on the behavior of phagocytes. Furthermore, the process of phagocytosis of ACs seems to be a unique, evolutionary conserved mechanism, since the anti-inflammatory effects of ACs are independent of species and the apoptotic stimulus (Voll et al., 1997).

3.3 Macrophage populations

MΦs are versatile immune cells which, depending on environmental stimuli, acquire diverse functional states. The extremes of this continuum are defined as the classically activated M1 and the alternatively activated M2 phenotype (Gordon, 2003). However, the alternative M2 definition does not satisfy and mirror the compelling evidence gathered over the last few years any more, since the M2 designation comprises MΦs with dramatic differences in their biochemistry and physiology (Edwards et al., 2006). Mosser and Edwards proposed a classification of MΦs according to their fundamental functions in host defense, wound healing and immune regulation (Mosser and Edwards, 2008) (see FIGURE 3.3). The authors assumed that there are many different ‘shades’ of MΦ activation and compared their classification to the three primary colours fusing into each other in a colour wheel. Thus, this should illustrate the ability of MΦs to exhibit characteristics, shared by more than one MΦ population.

3.3.1 M1 phenotype: classically activated or host defense macrophages

The M1 MΦ phenotype is established in response to stimuli, rapidly generated following infection or injury, like the bacterial cell wall component LPS and

interferon γ (IFNγ). M1 MΦs are characterized by enhanced microbicidal capacity, ensured by the production of pro-inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-6, IL-12, nitric oxide (NO) or ROS (Mackaness, 1964; O'Shea and Murray, 2008).

IFNγ can be produced by innate as well as adaptive immune cells; natural killer cells providing an early, generally transient innate source and T helper 1 (T_H1) cells a sustained adaptive one (Dale et al., 2008). Classical activation of MΦs requires Toll-like receptor (TLR) activation (e.g. by LPS) and subsequent MyD88-dependent TNF-α expression, which then cooperates with IFNγ in an autocrine way. In some cases exogenous IFNγ is dispensable, since some TLR ligands TRIF-dependently induce IFNβ production (Yamamoto et al., 2003). The importance of TLR activation and subsequent TNF-α production for the development of M1 MΦs was confirmed by experiments using eukaryotic parasites (*Leishmania* spp.) that failed to trigger TNF-α production in MΦs due to their lack of TLR ligands. Indeed, infected MΦs stimulated with exogenous TNF-α or with the TLR ligand LPS were able to clear the parasites completely (Bogdan and Rollinghoff, 1998).

Production of pro-inflammatory mediators is the main feature of classically activated MΦs, thus ensuring competent host defense. However, classical activation needs to be tightly controlled, since these factors may be detrimental by causing extensive host-tissue damage. In sepsis e.g., overproduction of pro-inflammatory cytokines induces a hyperinflammatory immune response often provoking severe tissue damage, organ failure, and death (Hotchkiss and Karl, 2003). Furthermore, IL-1 and IL-6, have been associated with the development and expansion of T_H17 cells (Bettelli et al., 2006), known for their strong efficient recruitment of polymorphonuclear leukocytes (PMN) and thereby contributing to pathological inflammatory autoimmune diseases, including rheumatoid arthritis (Szekanecz and Koch, 2007) and inflammatory bowel disease (Zhang and Mosser, 2008).

3.3.2 M2 phenotype: alternatively activated macrophages

Alternative activation of MΦs, e.g. with glucocorticoids, IL-4, IL-13 or IL-10, induces an anti-inflammatory M2 phenotype, which is characterized by attenuated production of pro-inflammatory, but enhanced secretion of anti-

inflammatory mediators such as IL-10, transforming growth factor β1 (TGF-β1) and prostaglandin E_2 (PGE_2). M2 designation regroups several populations of MΦs with different biochemistry and function, like wound-healing (M2a), hybrid (M2b) and regulatory (M2c) MΦs (Mantovani et al., 2004b; Mosser and Edwards, 2008).

3.3.2.1 M2a phenotype: wound-healing macrophages

Alternative activation of MΦs (M2) was originally described to depend on IL-4 and IL-13 stimulation. Basophils and mast cells are considered to be the main early sources of IL-4, which is thought to be the first innate signal released during tissue injury (Brandt et al., 2000; Loke et al., 2007). IL-4 induces arginase activity in MΦs, which converts L-arginine to L-ornithine, a precursor of polyamines and collagen, thereby promoting the production of extracellular matrix. Considering their ability to secrete extracellular matrix components and their implied function in wound healing, the classification of these cells was more recently specified as M2a or wound-healing MΦs (Mantovani et al., 2004b; Mosser and Edwards, 2008).

Besides innate signaling induced by tissue injury, adaptive T_H2 immune responses may also promote the development and maintainance of wound-healing MΦs, since IL-4 and IL-13 are the characteristic cytokines of T_H2 cells. *In vitro*, treatment of MΦs with IL-4 and/or IL-13 generates a population of M2a cells unable to present antigens to T cells, accompanied by attenuated production of pro-inflammatory cytokines as well as NO or ROS. Thus, these cells are less efficient at killing intracellular pathogens compared to classically activated MΦs (Edwards et al., 2006). Alongside the contribution to the production of extracellular matrix, wound-healing MΦs also show enhanced IL-10, mannose (MR) and scavenger receptor (SR) expression (Mantovani et al., 2004b; Stein et al., 1992). Furthermore, the polyamines produced by these cells influence the cytokine production and suppress clonal expansion of neighboring lymphocytes (Cordeiro-da-Silva et al., 2004).

Similar to dysregulation of M1 MΦs in autoimmunity, uncontrolled activation of wound-healing MΦs is detrimental for the host and has been associated with tissue fibrosis in chronic schistosomiasis as well as in asthma (Hesse et al., 2001; Munitz et al., 2008).

3.3.2.2 M2b phenotype: hybrid macrophages

M2b or hybrid MΦs result from exposure to immune complexes in combination with TLR or IL-1 receptor ligands. These MΦs have immunomodulating functions, since they induce consistent T_H2 responses. Like other alternative activated MΦs, they show enhanced IL-10 and impaired IL-12 expression, but on the contrary they still produce pro-inflammatory cytokines, like TNF-α, IL-6 and IL-1 (Mantovani et al., 2004b; Mosser and Edwards, 2008). M2b MΦs have been shown to originate from classically activated M1 MΦs, whose Fcγ receptor (FcγR) has been triggered by immune complexes. Thus, ligation of FcγR on M1 MΦs may invert the influence of an innate immune response, thereby deviating T_H1 into T_H2 responses. The authors postulated that this phenotypic switch might be exploited to modulate pathologies associated with T_H1 responses. Furthermore, they assumed that T_H2 responses following FcγR ligation might contribute to an improvement of immunoglobulin G (IgG) responses to weakly immunogenic antigens, since they observed that mice injected with M2b MΦs produced higher levels of IgG compared to M1 MΦs (Anderson and Mosser, 2002).

3.3.2.3 M2c phenotype: regulatory macrophages

Exposure of MΦs to environmental stimuli, such as glucocorticoides, PGs, immune complexes, IL-10 and ACs, generates M2c or regulatory MΦs (Mantovani et al., 2004b; Mosser and Edwards, 2008). Recently, further inducers, like adenosine (Hasko et al., 2007), S1P (Weigert et al., 2007) or adiponectin (Huang et al., 2008), have been suggested to provoke regulatory MΦ differentiation. This designation refers to their pronounced immune regulatory function due to secretion of high levels of the immunosuppressive mediators IL-10 and TGF-β1, and impaired production of IL-12 (Gerber and Mosser, 2001). Despite enhanced MR expression and the anti-inflammatory similarities, wound-healing and regulatory MΦs present clear functional and biochemical differences. In contrast to wound-healing MΦs (M2a), regulatory MΦs are neither impaired in antigen presentation nor do they contribute to the production of extracellular matrix components (Mosser and Edwards, 2008). Furthermore, regulatory MΦs play an important part in the late stages of adaptive immune responses, their main role consisting in dampening and

limiting inflammation in order to restore post-inflammation, but also post-traumatic tissue homeostasis (Bystrom et al., 2008; Mosser, 2003; Ruffell et al., 2009).

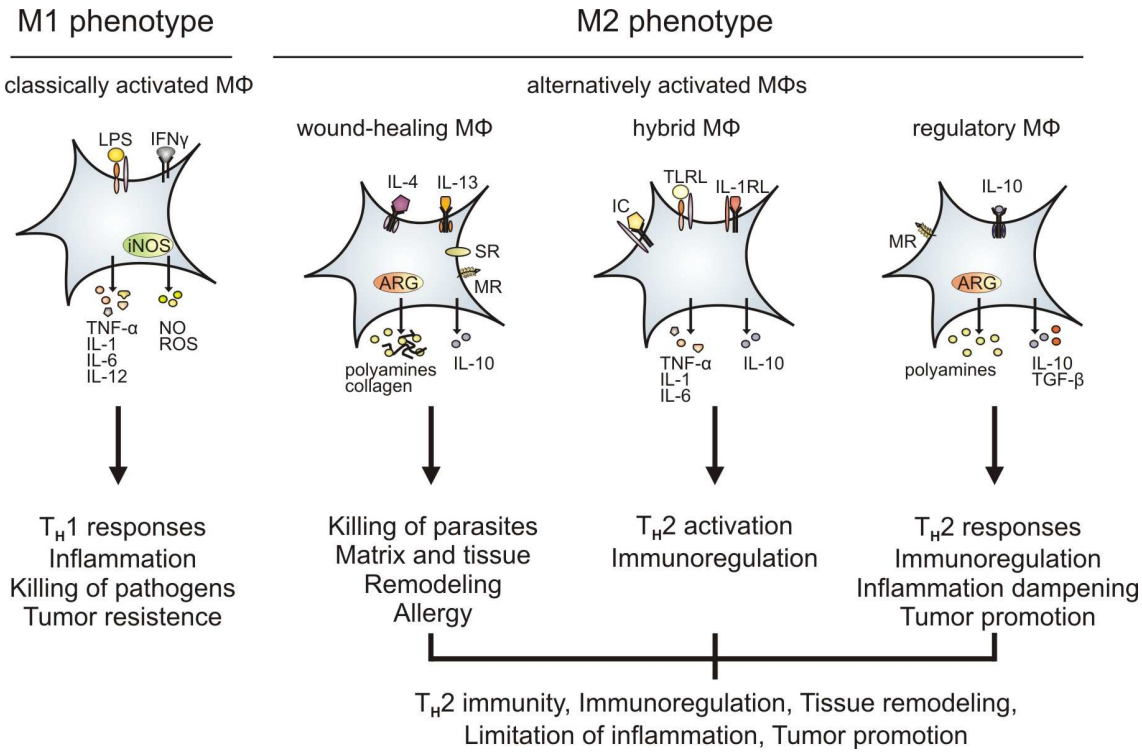

FIGURE 3.3: Macrophage populations.
The M1 macrophage (MΦ) phenotype is generated by classical activation via LPS and IFNγ stimulation. M1 MΦs produce cytotoxic nitric oxide (NO) via indicible NO synthase (iNOS) and reactive oxygen species (ROS) as well as pro-inflammatory mediators, like TNF-α, interleukin 1 (IL-1), IL-6 and IL-12. Alternative M2 MΦ activation is more heterogenous and raises three different M2 MΦ phenotypes. IL-4 and IL-13 induce wound-healing MΦs characterized by exibition of scavenger (SR) and mannose receptors (MR), release of immunosuppressive IL-10 and up-regulation of arginase (ARG) and consequent production of polyamines and the matrix component collagen. Hybrid MΦs are generated by stimulation with immune complexes (IC) and toll-like receptors ligands (TLRL) or IL-1 receptor ligands (IL-1RL), and they produce anti-inflammatory IL-10 as well as pro-inflammatory TNF-α, IL-1 and IL-6. Induction of regulatory MΦs is mediated by IL-10. This regulatory phenotype is characterized by ARG and MR expression and by release of immunoregulatory factors, like transforming growth factor β (TGF-β) and IL-10.

A growing body of evidence shows that regulatory MΦs may be exploited by parasites, viruses and bacteria (Benoit et al., 2008; Kim et al., 2008). These pathogens are able to mimic stimuli that induce this MΦ phenotype. For example, the Gram negative intracellular bacterium *Coxiella burnetti* causes host-cell apoptosis. Subsequent clearance of ACs by MΦs polarizes them into

regulatory cells with impaired bactericidal capacity and therefore tolerant towards intracellular bacterial growth (Benoit et al., 2008). Similar to this, intracellular parasites of *Leishmania spp.*, that primarily replicate in MΦs, escape host defense by polarizing the latter to an M2c phenotype. Indeed, these infected MΦs produce TGF-β and IL-10 and show impaired major histocompatibility complex II (MHCII) expression (Bogdan and Rollinghoff, 1998).

Worth mentioning is also the fact that tumor-associated MΦs (TAMs) may share some characteristics with regulatory MΦs (Pollard, 2008). The role of MΦs in cancer will be discussed in 3.3.3.

3.3.2.4 Role of extracellular signal-regulated kinase in regulatory macrophage phenotype

As mentioned above (see 3.3.2.3), there are many stimuli inducing an M2c MΦ phenotype, but the underlying mechanism still remains uncharacterized. Lucas *et al.* proposed the extracellular signal-regulated kinase (ERK) to be implicated in this phenotypic switch (Lucas et al., 2005). Previously, the same group had shown that LPS-mediated production of high levels of IL-12 was abrogated by co-ligation of the MΦ Fcγ receptor, accompanied by strong enhancement of IL-10 production (Gerber and Mosser, 2001). In a further study, Lucas *et al.* showed that LPS stimulation of MΦs in the presence of immune complexes (Fcγ receptor ligands) resulted in augmented ERK activation and subsequent phosphorylation-mediated histone modifications in the IL-10 promoter region. The high production of the immunosuppressive cytokine IL-10, which is one of the main features of regulatory MΦs, was prevented by pharmacolgical inhibition of ERK. Based on these observations, the authors suggested ERK signaling to mediate the observed phenotypic switch.

3.3.2.5 Role of cyclic adenosine monophosphate in regulatory macrophage phenotype

Recently, several reports suggested the potent immunomodulator cAMP to account for alternative activation of MΦs (Bystrom et al., 2008; Medeiros et al., 2009). Bystrom *et al.* suggested cyclic adenosine monophosphate (cAMP) signaling in MΦs to control their phenotype during resolution of inflammation. The authors isolated and characterized MΦs from the resolving phase of acute

inflammation and accordingly designated them as resolution-phase MΦs. In comparison to M1 MΦs, resolution-phase MΦs have far weaker bactericidal properties. Furthermore, they display an anti-inflammatory phenotype and express cyclooxygenase-2 (COX-2) as well as inducible nitric oxide synthase (iNOS) (Bystrom et al., 2008). Thus, the characteristics of these resolution-phase MΦs are very similar to those of regulatory MΦs, indicating that these MΦ populations are identical. Bystrom *et al.* convincingly showed that cAMP signaling is a prerequisite for the development of resolution-phase MΦs. Indeed, inhibition of the cAMP pathway transformed these cells into pro-inflammatory M1 MΦs. Inversely, elevation of cAMP in M1 cells converted them to resolution-phase MΦs (Bystrom et al., 2008). Another study further emphasizes the importance of cAMP in regulatory MΦs. Medeiros *et al.* showed that COX-dependent PGE_2 production upon clearance of ACs potently prevented disposal of pathogens *in vivo* by alveolar MΦs, which secreted high levels of IL-10. Furthermore, they attributed these observations to PGE_2/E prostanoid receptor 2 (EP2) signaling and subsequent adenylyl cyclase-mediated cAMP production (Medeiros et al., 2009). These observations indicate that cAMP might participate in the polarization of regulatory MΦs.

3.3.3 M2 macrophages in cancer

Taking into consideration that MΦs discern modified ‘self’ molecules, like e.g. ACAMPs on ACs and thereupon clear them, it seems logical to postulate recognition and elimination of modified tumor cells as well. Unfortunately, many studies show that this assumption is not the case. On the contrary, TAMs seem to aquire biochemical properties promoting tumor growth and progression (Lewis and Pollard, 2006). Their presence in solid tumors, which can attain 50% of tumor mass in breast cancer e.g. (Murdoch et al., 2004), has even been correlated with poor survival prognosis for patients (Bingle et al., 2002).

However, in early stages of cancer, MΦs display a pro-inflammatory M1-like phenotype. On the one hand, these classically activated MΦs were shown to be cytotoxic towards tumor cells, thereby contributing to early eradication of transformed cells (Romieu-Mourez et al., 2006). On the other hand, M1 MΦs were reported to contribute to early neoplasia by producing free radicals, thus inducing DNA damage that causes mutations and predisposes cells to

transformation (Swann et al., 2008). Even though opinions on their role are still controversial, there is a general agreement about their pro-inflammatory phenotype.

During tumor progression and growth, tumor-derived agents induce pronounced changes in the physiology of MΦs thereby promoting the development of regulatory cells (Pollard, 2008) with impaired production of tumoricidal factors such as TNF-α and NO. Like regulatory MΦs, these TAMs produce high levels of IL-10 and little IL-12. They can also inhibit immune responses to neo-antigens and prevent activation of neighboring MΦs (Biswas et al., 2006). Furthermore, a recent study suggested that regulatory MΦs promote tumor growth by contributing to angiogenesis (Lin et al., 2006). The importance of TAMs in vascular development, and consequently for tumor malignancy, was further strengthened in a breast cancer mouse model. Indeed, preventing infiltration of MΦs into the tumor retarded its development due to defective vascularization (Lin et al., 2006). Along this line, infiltration of TAMs was also correlated with high blood vessel density in tumors (Yu and Rak, 2003). Taken together, these observations convincingly suggest that TAMs promote tumor survival, growth, angiogenesis and metastasis formation as well as immune evasion.

Candidate inducers of M2-like regulatory polarization within the tumor microenvironment include IL-4, IL-10, TGF-β, PGs, hypoxia and ACs (Knowles and Harris, 2001; Liu et al., 2001; Weigert et al., 2007). Lately the assumption arose that tumors mis-use the physiological function of MΦs in the resolution of inflammation and re-establishment of tissue homeostasis by polarizing them in an AC-similar way (Mantovani et al., 2004a; Weigert et al., 2007). This assumption was based on studies showing that tumor regression could be induced *in vivo* by preventing MΦ and dendritic cell-mediated AC-recognition (Bondanza et al., 2004), and that MΦ cytotoxicity towards viable tumor cells could be impaired by addition of apoptotic tumor cells (Reiter et al., 1999).

3.4 Macrophage polarization by apoptotic cells

Formerly, disposal of ACs was thought to be an immunologically neutral process, since no release of pro-inflammatory cytokines was observed (Stern et al., 1996). The absence of an inflammatory response was attributed to a fast

phagocytosis of ACs before they could release harmful cellular components in the surrounding tissue by loss of membrane integrity (Savill et al., 1993). However, it was shown that inducers of apoptosis, like e.g. ultraviolet radiation, could alleviate inflammatory diseases (Abel, 1995). Thus, the question arose whether ACs only fail to induce inflammation, or whether they also actively suppress the inflammatory response.

3.4.1 Attenuated pro- and enhanced anti-inflammatory response of macrophages towards apoptotic cells

Studies in various model systems showed that phagocytosis of ACs is an active, immunosuppressive and anti-inflammatory process. In 1997, Voll *et al.* reported that co-incubation of apoptotic lymphocytes with LPS-elicited monocytes attenuated pro-inflammatory cytokine production of TNF-α and IL-1β and increased the release of the anti-inflammatory mediator IL-10 from the latter (Voll et al., 1997). Beyond that, a study of Fadok *et al.* showed that ACs induce secretion of TGF-β1, PGE_2 and platelet activating factor (PAF) in MΦs probably via PtdSer exposure. Furthermore, autocrine signaling of TGF-β1, PGE_2 and PAF contributed to impaired secretion of pro-inflammatory cytokines, like TNF-α, IL-1β and IL-8. On the one hand, addition of exogenous TGF-β1, PGE_2 or PAF prevented LPS-mediated cytokine production. On the other hand LPS-mediated cytokine production in MΦs, which had phagocyted ACs, could be restored by scavenging TGF-β with TGF-β neutralizing antibodies, blocking COX by indomethacin or by PAF receptor antagonists (Fadok et al., 1998). In this context, TGF-β1 was described to impair pro-inflammatory cytokine release by activating ERK, provoking up-regulation of mitogen-activated protein kinase (MAPK) phosphatase 1 and subsequent inhibition of p38-MAPK and nuclear factor κB (NF-κB) (Xiao et al., 2002). Reduced cytokine expression is generally attributed to inhibition of NF-κB, since this transcription factor is the major transcriptional regulator of cytokine expression (Bonizzi and Karin, 2004). Besides the potential role of TGF-β, the tyrosine kinase receptor Mer also seems to be an appropriate candidate mediating inhibition of NF-κB. In fact, AC-dependent inhibition of NF-κB in phagocytes could be prevented by inhibiting or knocking down the tyrosine kinase receptor Mer as well as by overexpressing a Mer tyrosine kinase-dead mutant (Sen et al., 2007; Tibrewal et al., 2008). This

points to the implication of PtdSer in this process, since MΦ Mer kinase has been reported to bind to PtdSer on ACs via the bridging molecule Gas6 (Wu et al., 2006). In addition to TGF-β and Mer tyrosine kinase-dependent late regulation (≥ 18 hours), co-activators and co-repressors have been described to modulate NF-κB activity at earlier times (Cvetanovic and Ucker, 2004). Recently, Jennewein *et al.* reported that AC-mediated early inhibition (≥ 1.5 hours) of NF-κB resulted from the interplay between peroxisome proliferator-activated receptor γ (PPARγ) and the nuclear receptor co-repressor (NCoR). NCoR, a co-repressor binding to NF-κB sites under basal conditions, is degraded upon LPS stimulation via the ubiquitination/19S proteasome pathway, followed by binding of co-activators and initiation of transcription of pro-inflammatory mediators (Pascual et al., 2005). AC-dependent PPARγ sumoylation in MΦs directs it to NCoR, thereby inhibiting proteasomal degradation and subsequent release of active NF-κB, thus transrepressing NF-κB-mediated promoter activities of pro-inflammatory cytokines (Jennewein et al., 2008). Interestingly, another study proposed p50 homodimerization to be crucial for NF-κB inhibition in tumor-associated MΦs (Saccani et al., 2006), suggesting that depending on environmental stimuli, distinct signaling pathways are initiated, yet with a similar outcome.

Furthermore, *in vivo* experiments on pneumonias suggested AC-dependent TGF-β1 secretion from MΦs to have anti-inflammatory effects (Huynh et al., 2002). Huynh *et al.* also argued for PtdSer-dependent TGF-β1 release since human monomyelocytes, which did not expose PtdSer on the outer leaflet of the plasma membrane upon apoptosis, were unable to induce TGF-β1 secretion. Along this line, MΦs did not release TGF-β1, when incubated with irradiated tumor cells on which PtdSer had been masked with annexin V (Bondanza et al., 2004). Another more recent study supports the assumption that PtdSer receptor activation signals towards TGF-β production. The authors induced TGF-β1 secretion by activating the novel PtdSer receptor Stabilin-2 with anti-Stabilin-2 antibodies (Park et al., 2008). Although the phagocyte receptor mediating TGF-β1 secretion is still uncharacterized, transcriptional regulation by MAPKs (p38, ERK and Jnk) as well as translational modulation by Rho/PI3K/Akt/mTor seem to be involved (Xiao et al., 2008).

Worth mentioning is also the fact that phagocytosis *per se* is not required to suppress inflammation. Knock-down studies on CD14 as well as CD36/VnR deficient mice showed that, despite impaired phagocytosis of ACs, secretion of TGF-β was still elevated and TNF-α release was reduced (Devitt et al., 2004; Lucas et al., 2006).

3.4.2 Attenuated nitric oxide and reactive oxygen species formation in macrophages in response to apoptotic cells

MΦs respond to bacteria or bacterial components with an up-regulation of ROS and NO production, which mediates in part their bactericidal capacity (Bogdan, 2001; Forman and Torres, 2002). Cytotoxic ROS formation in MΦs, the so-called oxidative burst is triggered by PKCα-mediated phosphorylation of $p47^{phox}$, activation of Rac1/2 and assembly of the NADPH oxidase complex (Lambeth, 2004; Larsen et al., 2000). NO production in response to LPS and/or IFN-γ is initiated by up-regulation of the iNOS that oxidizes L-arginine to L-citrulline and NO (Bogdan, 2001) (see FIGURE 3.4).

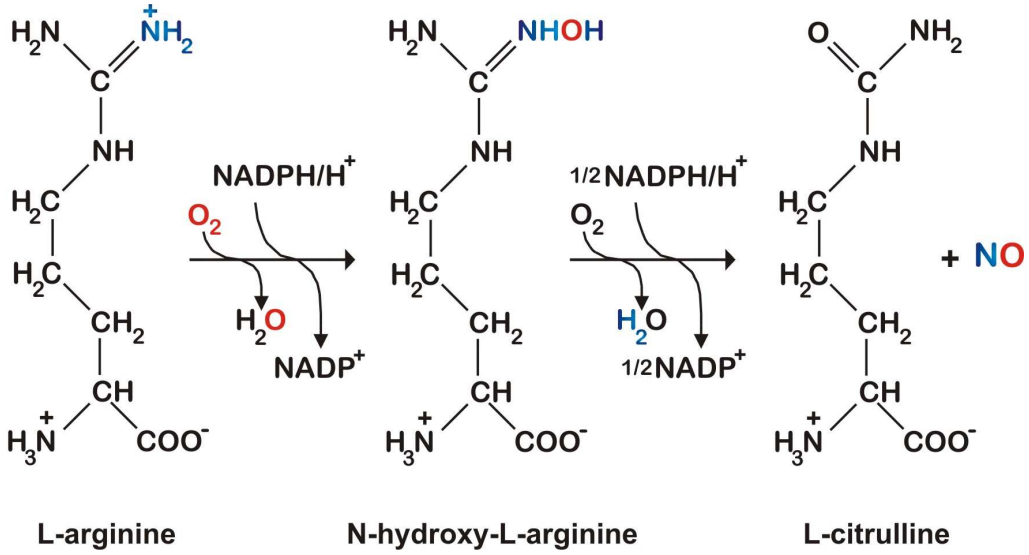

FIGURE 3.4: Scheme of the reaction catalyzed by nitric oxide synthase (NOS). The substrate of the enzyme NOS (which exists in several isoforms) is the aminoacid L-arginine. This aminoacid is hydroxylated twice into nitric oxide (NO) and L-citrulline.

Several studies reported the ability of ACs to impair ROS as well as NO formation in MΦs (Freire-de-Lima et al., 2000; Johann et al., 2006; Serinkan et al., 2005). Serinkan *et al.* observed that apoptotic Jurkat cells as well as PtdSer-enriched viable Jurkat cells inhibited ROS production in MΦs in a cell-cell-

contact dependent way, thus pointing to a PtdSer-triggered mechanism (Serinkan et al., 2005). Furthermore, Johann *et al.* suggested AC-mediated activation of PPARγ to account for inhibition of ROS formation (Johann et al., 2006). Along this line, activation of PPARγ has been shown to interfere with PKCα translocation to the plasma membrane by a direct protein-protein interaction, thereby abolishing PKCα-mediated phosphorylation of $p47^{phox}$, which is indispensable for NADPH oxidase assembly and subsequent ROS production (von Knethen et al., 2007). Concerning AC-dependent impairment of NO formation, enhanced arginase (ARG) expression seems to play a decisive role. ARG I and ARG II compete with iNOS for the common substrate by metabolizing L-arginine to urea and ornithine (Johann et al., 2007; Morris, 2004; Topal et al., 2006) (see FIGURE 3.5).

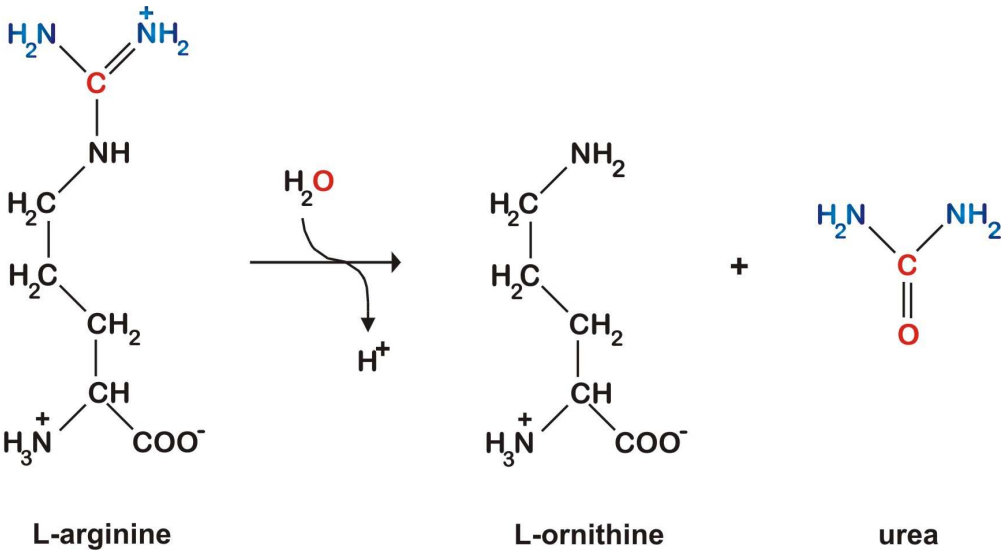

FIGURE 3.5: Scheme of the reaction catalyzed by arginase (ARG).
The substrate of the enzyme ARG, which exists in several isoforms, is the aminoacid L-arginine. This aminoacid is hydrolyzed into L-ornithine and urea.

Johann *et al.* proposed that impaired NO production resulted from TGF-β-independent up-regulation of ARG II and unaltered iNOS expression (Johann et al., 2007), while others suggested a TGF-β/PtdSer-dependent up-regulation of ARG I and simultaneous down-regulation of iNOS to account for this inhibition (Freire-de-Lima et al., 2006). However, TGF-β-dependent ARG I up-regulation occurred late (≥ 24 hours) in comparison to inhibition of NO production, pointing to an early TGF-β-independent and a late, at least in part, TGF-β-mediated inhibition.

3.4.3 Impact of soluble factors released from apoptotic cells on macrophages

AC-dependent polarization of MΦs was thought to require receptor-ligand interactions or phagocytosis-derived signals, but more recent studies show that ACs confer immunomodulation by releasing soluble factors. ACs exert direct anti-inflammatory effects by secreting factors with immunosuppressive properties, like the cytokines IL-4 (Hodge et al., 2002), IL-10 (Gao et al., 1998) and TGF-β (Chen et al., 2001; Hodge et al., 2002) or the lipid mediator sphingosine-1-phosphate (S1P) (Weigert et al., 2006).

3.4.3.1 Effects of IL-4, IL-10 and TGF-β on macrophages

Besides IL-4 release from ACs, IL-4 is primarily produced by basophils and mast cells in response to tissue injury (Brandt et al., 2000). IL-4 rapidly shifts the MΦ phenotype towards wound healing. These MΦs fail to present antigens to T cells and are impaired in the production of pro-inflammatory cytokines as well as ROS and NO (Edwards et al., 2006).

IL-10 is a potent anti-inflammatory cytokine, mainly secreted by regulatory T cells, but also by ACs and MΦs themselves. Kuhn *et al.* reported IL-10 to be immunosuppressive, since IL-10-deficient mice seem unable to constrict inflammation and eventually develop chronic intestinal inflammation (Kuhn et al., 1993). In accordance, Gao *et al.* showed that Fas-triggered apoptotic lymphoid cells rapidly produce IL-10, which promotes antigen presenting cell (APC)-dependent T_H2 cell differentiation, whereas apoptotic IL-10-deficient cells, as well as living cells, favor T_H1 induction (Gao et al., 1998). Furthermore, among other stimuli (e.g. PGE_2) (Strassmann et al., 1994), IL-10 was reported to induce a population of MΦs that produced high levels of IL-10 (Martinez et al., 2008). Paracrine and/or autocrine IL-10 signaling then suppresses transcription of pro-inflammatory mediators in a STAT3-dependent manner (O'Shea and Murray, 2008).

A further anti-inflammatory feature of ACs consists in their ability to secrete TGF-β. After disruption of the mitochondrial membrane potential in ACs, the TGF-β stock is redistributed into the cytosol and subsequently secreted in the surrounding tissue (Chen et al., 2001). The immunosuppressive properties of

TGF-β consist in attenuated pro-inflammatory cytokine, ROS and NO production, and have been discussed extensively in 3.4.1 and 3.4.2.

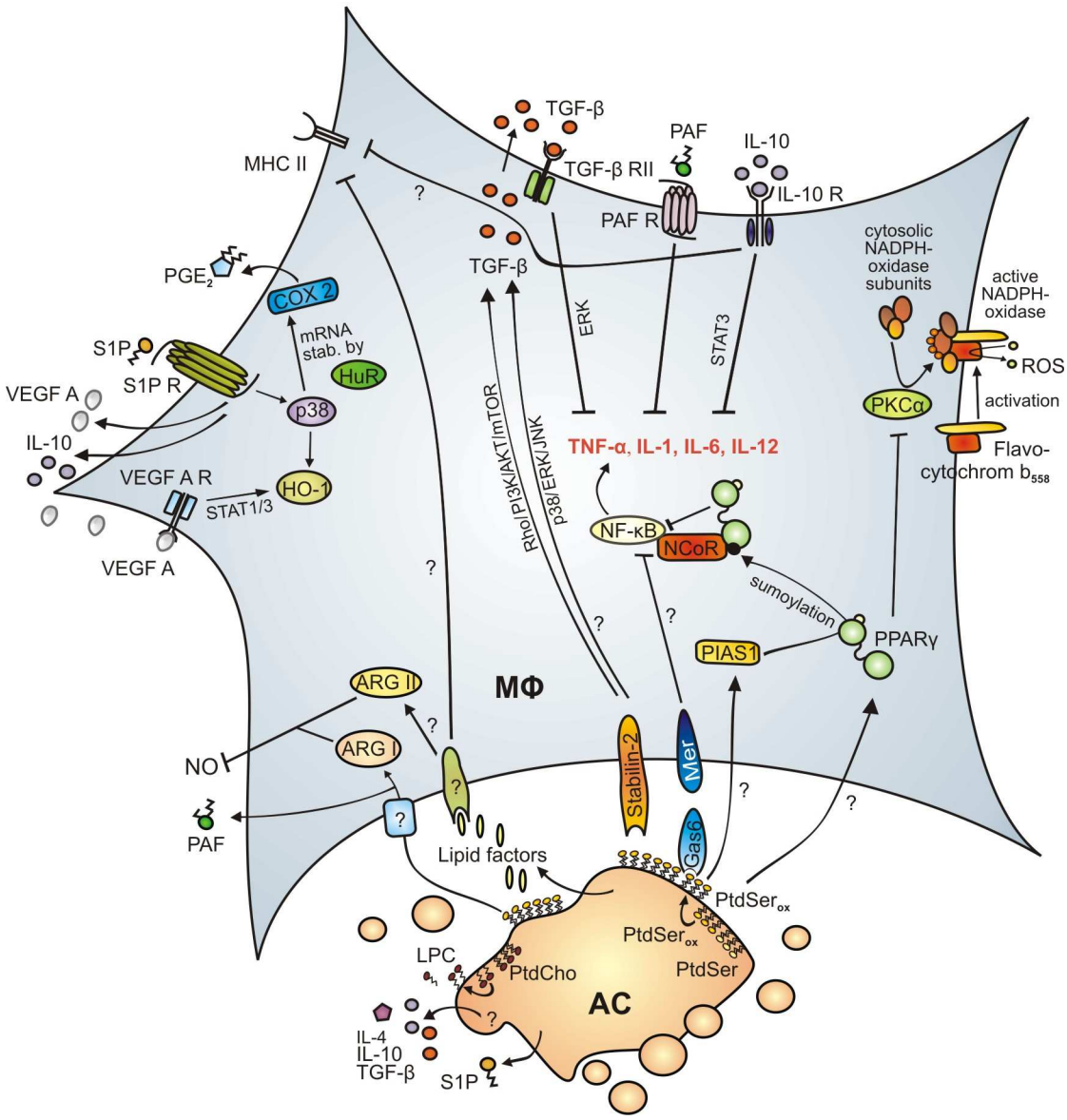

FIGURE 3.6: Apoptotic cell-mediated polarization of macrophages.
Apoptotic cells (ACs) secrete interleukin 4 (IL-4), IL-10, transforming growth factor β (TGF-β) and lipid factors, like e.g. lysophosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P) and expose oxidized phosphatidylserine ($PtdSer_{ox}$) on the outer leaflet of the plasma membrane, which induce polarization of macrophages (MΦs). Some unidentified lipid factor and PtdSer induce up-regulation of arginase II (ARG II) and ARG I respectively in MΦs, thereby impairing cytotoxic nitric oxide (NO) production. Anti-inflammatory S1P mediates its actions by binding to S1P receptors (S1P R) and subsequently provoking vascular endothelial growth factor A (VEGF A) and IL-10 release as well as p38 activation, which induces heme oxygenase 1 (HO-1) expression. Then, VEGF A autocrinely binds to the VEGF A receptor (VEGF A R) and also contributes to up-regulation of HO-1 in a STAT1/3-dependent way. Furthermore, S1P-mediated activation of p38 provokes HuR-dependent stabilization of cyclooxygenase 2 (COX 2) mRNA leading to production/secretion of prostaglandin E_2

(PGE_2). PtdSer activates Stabilin-2 on MΦs, which induces production/secretion of TGF-β, as well as another, yet unidentified, receptor provoking release of platelet activating factor (PAF). TGF-β, PAF and IL-10 bind to their respective receptors on MΦs, thereby inhibiting the production of pro-inflammatory mediators, like tumor necrosis factor α (TNF-α), IL-1, IL-6 and IL-12. In addition, PtdSer also activates Mer via the bridge molecule Gas6, thereby inhibiting nuclear factor κB (NF-κB)-mediated induction of pro-inflammatory cytokines. Activation of PIAS1/sumoylated peroxisome proliferator-activated receptor γ (PPARγ)/nuclear receptor co-repressor (NCoR) is a further mechanism by which PtdSer inhibits NF-κB. Activated PPARγ also impairs reactive oxygen species (ROS) production by preventing PKCα-dependent activation of NADPH-oxidase. Finally, IL-10 as well as lipid factors secreted by ACs might inhibit the expression of major histocompatibility complex II (MHC II) in MΦs. '?' designates putative/uncharacterized pathways.

3.4.3.2 Effect of S1P on macrophages

Besides the anti-inflammatory cytokines IL-4, IL-10 and TGF-β, ACs also secrete the bioactive lipid mediator S1P (Weigert et al., 2006). S1P emanates from the sphingolipid metabolic cascade (see FIGURE 3.7), starting with the hydrolysis of sphingomyelin by sphingomyelinase (SMase), which produces ceramide. Thereupon, ceramide can be deacylated by ceramidase (CDase) to sphingosine, and sphingosine is phosphorylated by sphingosine kinase (SK) to S1P. Furthermore, S1P may also be synthesized *de novo*, starting with the condensation of palmitoyl-CoA and serine to 3-ketosphinganine, which is then converted into ceramide by several reactions. S1P turnover is mediated by reversible dephosphorylation to sphingosine by S1P phosphatase (SPP) or by irrevocable degradation through S1P lyase (SPL) (Le Stunff et al., 2004).

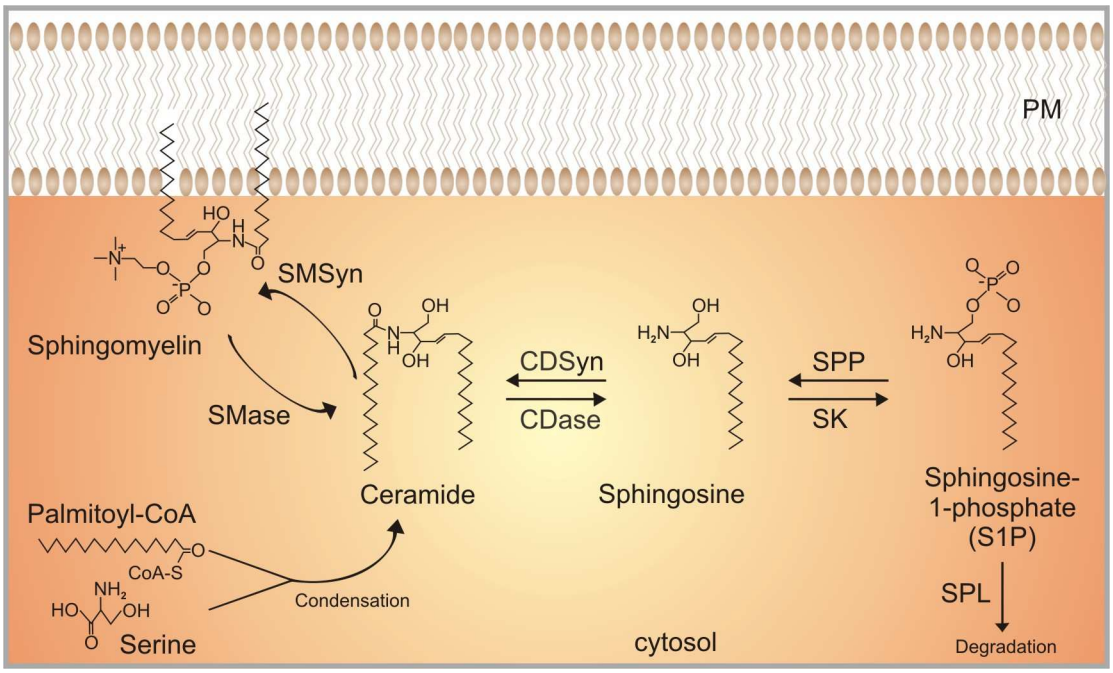

FIGURE 3.7: Sphingolipid metabolism.
Sphingomyelin is hydrolized by sphingomyelinase (SMase) to ceramide. Ceramide can be transformed to sphingosine by ceramidase (CDase) or re-converted to sphingomyelin by spingomyelin synthase (SMSyn). Sphingosine is phosphorylated by sphingosine kinase (SK) to sphingosine-1-phosphate (S1P) or re-transformed to ceramide by ceramide synthase (CDSyn). S1P can be hydrolized to sphingosine by S1P phosphohydrolase (SPP) or irreversibly degraded by S1P lyase (SPL). S1P may also be synthesized *de novo* through the condensation of serine with palmitoyl-CoA.

S1P can be exported from cells via ABC transporters (Mitra et al., 2006), but it can also be produced extra-cellularly by secreted SK1 and SK2 (Venkataraman et al., 2006)(Weigert et al., unpublished data), where it signals by binding to five specific G protein-coupled receptors (GPCRs) ($S1P_{1-5}$). S1P signaling via $S1P_{1-5}$ has been linked to a series of important events, like vascular development and maturation, cardiac development, directed cell motility and immunity (Spiegel and Milstien, 2003). S1P receptors and corresponding down-stream effector kinases are summarized in FIGURE 3.8.

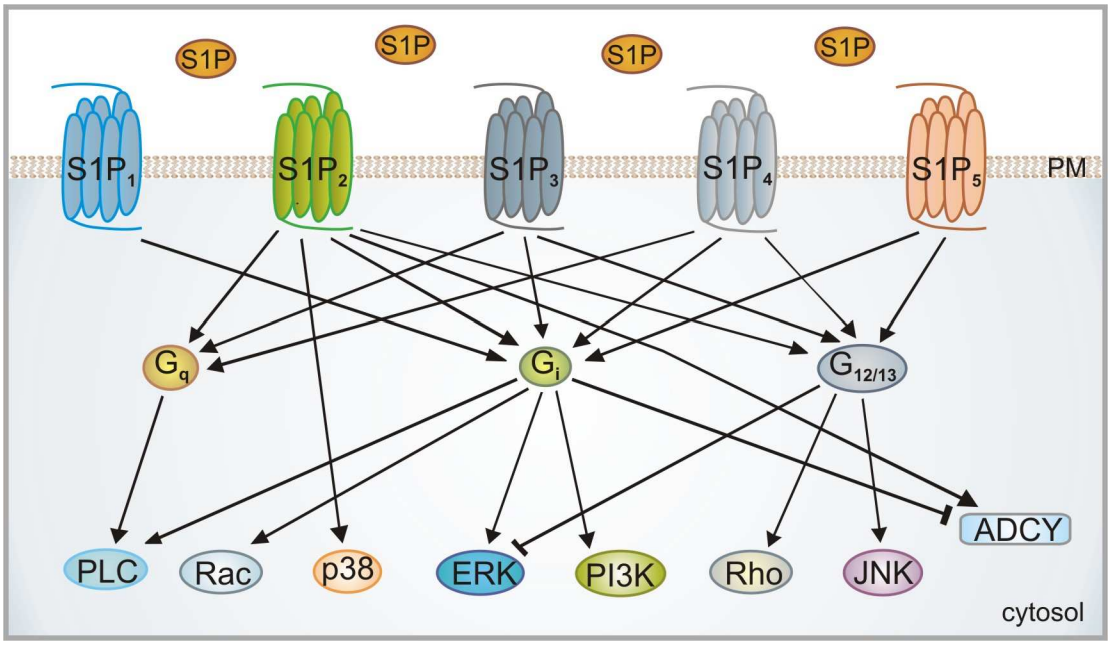

FIGURE 3.8: Sphingosine-1-phosphate receptors and effectors.
Exported sphingosine-1-phosphate (S1P) binds to five specific receptors ($S1P_{1\text{-}5}$) and triggers many signaling pathways either directly or by activating G proteins, like G_q, G_i or $G_{12/13}$. Phospholipase C (PLC), Rac, p38, extracellular signal-regulated kinases (ERK), phosphatidylinositol-3 kinase (PI3K), Rho, jun N-terminal kinase (JNK) or adenylyl cyclase (ADCY) constitute some examples of effectors mediating these signaling pathways. (PM: plasma membrane)

Several studies documented the anti-inflammatory (Idzko et al., 2002; Xin et al., 2004) and anti-apoptotic effects of S1P (Gomez-Munoz et al., 2003; Weigert et al., 2007). Weigert *et al.* showed that AC-derived S1P protects MΦs from apoptosis, enhances IL-10 and IL-8 expression and attenuates TNF-α as well as IL-12p70 release. These anti-apoptotic and anti-inflammatory effects of S1P might be mediated, at least in part, by up-regulation of heme oxygenase 1 (HO-1) (Weis et al., 2009). The authors described a biphasic induction of HO-1, the early one (6 hours) being S1P receptor 1/p38-dependent, whereas the second one (24 hours) was attributed to autocrine signaling of vascular endothelial growth factor A (VEGF-A). HO-1, which metabolizes heme to biliverdin, ferrous iron and carbon monoxide, has been described as anti-inflammatory (Deshane et al., 2005; Kim et al., 2006; Otterbein et al., 2000) and anti-apoptotic by up-regulating Bcl-2 and Bcl-X_L (Weis et al., 2009). Furthermore, tumor promoting properties have recently been attributed to S1P and TGF-β secreted by apoptotic tumor cells. In combination with other AC-derived factors, S1P and TGF-β initiate a HIF-1 response in MΦs under normoxic conditions resulting in

e.g. VEGF release. Based on experiments with MΦs from conditional HIF-1α knockout mice, Herr *et al.* showed that upon stimulation with supernatants of ACs, MΦs HIF-1-dependently produce factors that induce CD31 expression in murine embryonic stem cells (Herr et al., 2009).

3.5 Cyclic adenosine monophosphate signaling pathways

Upon stimulation of a G protein-coupled receptor (GPCR), the associated heterotrimeric G protein exchanges guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α-subunit, which is thereby activated and dissociates from the βγ-dimer. Then, both the α- and βγ-subunits can initiate or inhibit distinct signaling pathways. Termination of GPCR-mediated signaling cascades is achieved by the intrinsic GTPase activity of the α-subunit, which hydrolyzes GTP to GDP and then re-associates with the βγ-dimer (Pierce et al., 2002).

The activated α-subunit of the G_s subtype induces e.g. the conversion of adenosine triphosphate (ATP) into cAMP and pyrophosphate (PP_i) by activating the lyase adenylyl cyclase (Levitzki, 1988). The intracellular second messenger cAMP then binds and activates effector or regulator molecules (see FIGURE 3.9) such as cAMP-gated ion channels (Kaupp and Seifert, 2002), guanine nucleotide-exchange proteins directly activated by cAMP (EPACs) (Bos, 2003), phosphodiesterases (PDEs) (Houslay and Adams, 2003) and protein kinase A (PKA) (Walsh et al., 1968). Termination of cAMP signaling is ensured by PDE activity, which hydrolyzes cAMP to AMP (Houslay and Adams, 2003). The main intracellular target of cAMP is PKA, a tetrameric holoenzyme consisting of two catalytic (C) subunits and a regulatory (R) subunit dimer. Upon cAMP binding to the R-subunits, the active C-subunits dissociate and target down-stream effectors (Corbin et al., 1973; Potter and Taylor, 1979). As broad-spectrum serine/threonine kinases, the C-subunits potentially phosphorylate numerous substrates, among which the transcription factor cAMP responsive element binding protein (CREB) is the most prominent (Shaywitz and Greenberg, 1999) (see FIGURE 3.9).

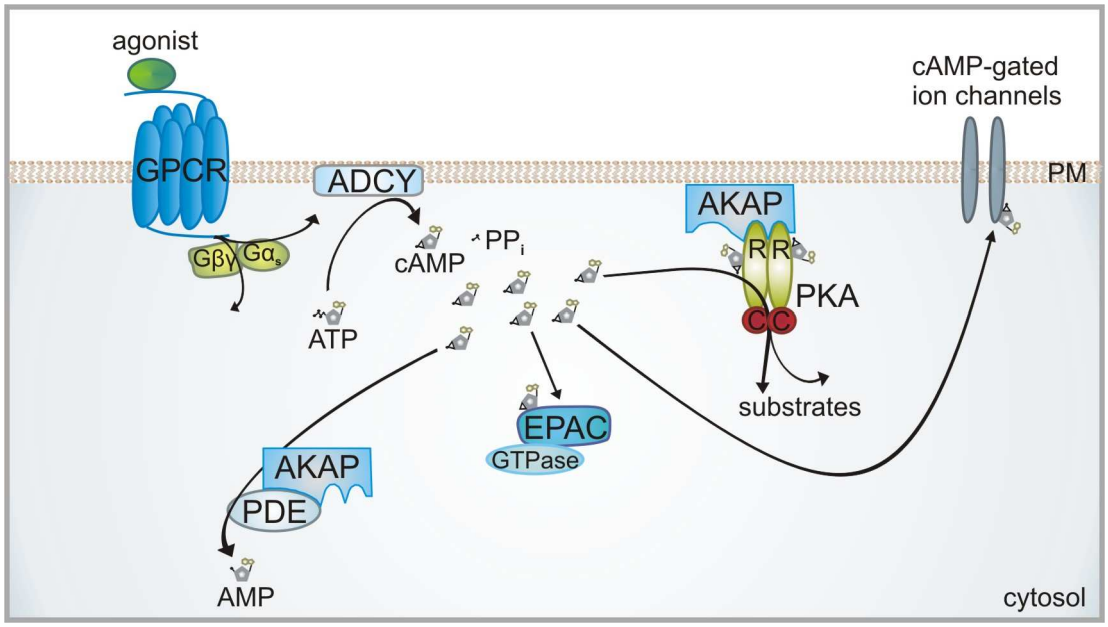

FIGURE 3.9: Cyclic adenosine monophosphate signaling pathways.
Agonist-binding to a G protein-coupled receptor (GPCR) provokes the dissociation of the heterotrimeric G protein allowing the $G\alpha_s$-subunit to activate adenylyl cyclase (ADCY), which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and pyrophosphate (PP_i). The actions of the second messenger cAMP are mediated by targets, like cAMP-gated ion channels, exchange proteins directly activated by cAMP (EPAC) and activated catalytic subunits of protein kinase A (PKA). cAMP is eventually hydrolized by phosphodiesterase (PDE) to adenosine monophosphate (AMP). PKA and PDE are found mostly bound to A kinase-anchoring proteins (AKAP).

In order to achieve specific signaling, the PKA holoenzyme has to be compartmentalized to allow phosphorylation of only a subset of potential targets. This confinement is mediated by signal-organizing molecules, so-called A kinase-anchoring proteins (AKAPs), which bind and direct PKA to distinct subcellular locations (Carnegie and Scott, 2003). Along this line, in opposition to the former assumption that cAMP diffused freely in the cytosol (Kasai and Petersen, 1994), this second messenger was suggested to accumulate at specific sites within cells. In fact, live-cell imaging of rat neonatal cardiac myocytes clearly visualized gradients rather then a uniform intracellular distribution of cAMP (Zaccolo and Pozzan, 2002).

3.5.1 Properties of A kinase-anchoring proteins

The AKAP family is constituted of structurally diverse members, although sharing common motifs. They all contain a PKA-anchoring domain and a localization signal targeting them to specific subcellular compartments. Besides

PKA, they also bind other signaling molecules. The colocalization of PKA with other signaling enzymes might be the main feature of AKAPs. These multivalent scaffold proteins often unite enzymes for both signal transduction and signal termination, thereby creating a regulatory site for a determined signaling process (Kapiloff, 2002; Zaccolo et al., 2002).

The muscle-specific AKAP (mAKAP) is one example of an anchoring protein containing both regulators of cAMP concentration and effectors of cAMP actions, which results in a local modulation of PKA activation. The mAKAP complex consists, among others, of PKA and PDE4D3, the latter being an adaptor protein recruiting ERK5 and EPAC1.

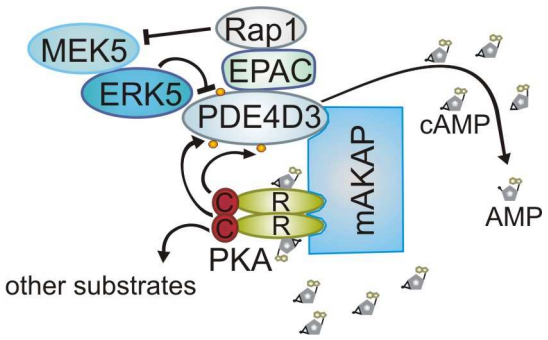

FIGURE 3.10: Constitution of muscle A kinase-anchoring protein.
Muscle A kinase-anchoring protein (mAKAP) binds cyclic adenosine monophophate (cAMP)-dependent protein kinase A (PKA) and cAMP-hydrolizing phosphodiesterase (PDE) and holds them in near proximity. In a feed-back inhibition mechanism, cAMP-activated PKA phosphorylates PDE4D3 twice, thereby enhancing its activity and consequently cAMP hydrolysis. However, PDE4D3 is also an adaptor protein for extracellular signal-regulated kinase 5 (ERK5) and exchange proteins directly activated by cAMP (EPAC). ERK5-dependent phosphorylation inhibits the PDE4D3 activity, while high cAMP concentrations activate EPAC/Rap1, which inhibits mitogen-activated protein kinase kinase 5 (MEK5), the up-stream kinase of ERK5. Thus, the mAKAP complex finely tunes cAMP-dependent cellular responses.

In a feed-back inhibition mechanism, activated PKA phosphorylates PDE4D3 on serine 13 and serine 54, thereby increasing the binding affinity for mAKAP as well as the catalytic activity of PDE4D3. In addition, ERK5 represents a further regulatory module, since this kinase phosphorylates PDE4D3 on serine 579, which decreases phosphodiesterase activity and subsequently increases cAMP levels. However, sustained cAMP concentrations activate EPAC1 and consequently Rap1, resulting in the inhibition of ERK5. Dodge-Kafka *et al.* showed that PDE4D3 was the key regulatory enzyme in the mAKAP complex,

since the dynamic cAMP signaling depended on the phosphorylation state of PDE4D3 (Dodge-Kafka et al., 2005). Thus, the mAKAP complex is an integrated and internally regulated network allowing a spatio-temporal control of cAMP signaling (see FIGURE 3.10).

3.6 Regulation of arginase II expression

There are two ARG isoforms in mammals (Morris, 2004), which catalyze the hydrolysis of L-arginine to L-ornithine and urea. Although their amino acid sequences are 58% identical and their enzymatic properties are similar, transcriptional regulation as well as their tissue, cellular and subcellular distributions are different. The hepatic isoform, ARG I is mainly expressed in the cytosol of hepatocytes and catalyzes the last step of urea synthesis. In contrast, ARG II is located in mitochondria, has wide tissue distribution, with expression in kidney, brain, small intestine and in MΦs (Grody et al., 1987; Morris et al., 1998; Vockley et al., 1996). Because of its close proximity to ornithine aminotransferase in mitochondria, ARG II is considered to be involved primarily in the production of L-ornithine as a precursor of proline (Morris, 2004). Even though the two ARG isoforms mostly occur individually, some cell types like MΦs express both of them (Barksdale et al., 2004; Morris et al., 1998). Based on complex expression patterns, Morris *et al.* suggested that ARG isoforms may play distinct, partially overlapping functional roles in MΦ arginine metabolism. Furthermore, ARG expression in MΦs modulates NO production through competition with iNOS (Freire-de-Lima et al., 2006; Gotoh and Mori, 1999; Johann et al., 2007; Morris, 2004; Topal et al., 2006). Despite different opinions on the mechanism accounting for ARG-dependent impaired NO formation, ARG II seems to account for an early and ARG I probably for a late NO modulation (see 3.4.2). Another example of differential ARG I and ARG II regulation was provided by Barksdale *et al.*, who investigated the effect of mediators released during trauma, such as catecholamines, TGF-β and T_H2 cytokines on ARG expression in MΦs. The authors observed that, on the one hand isoproterenol synergized with IL-4, IL-13, and TGF-β to increase ARG I and on the other with IL-10 to up-regulate ARG II in RAW264.7 MΦs. They proposed these synergistic mechanisms to contribute to cellular

immunosuppression observed after trauma by impairing NO production (Barksdale et al., 2004).

Marathe *et al.* reported that in MΦ cell lines as well as primary MΦs, ARG II is a direct target of liver X receptor (LXR), a transcription factor mediating anti-inflammatory actions (Marathe et al., 2006). Along this line ACs, which are known to polarize MΦs towards an anti-inflammatory regulatory phenotype, have also been reported to secrete factors that up-regulate ARG II in MΦs (Johann et al., 2007).

As a critical enzyme in polyamine biosythesis, ARG II has been linked to proliferation and/or tissue repair in rheumatoid arthritis, since it was expressed in synovial fluid cells from patients with different forms of arthritis. Furthermore, stimulation of these cells with dibutyryl-cAMP, PGE_2 and LPS further up-regulated ARG II (Corraliza and Moncada, 2002). ARG II was also shown to participate in anti-viral defense by providing the precursor for spermine biosynthesis, which is known to possess anti-viral activity and to mediate apoptosis. In fact, ARG II protein and enzymatic activity were induced in Sendai virus-infected Jurkat cells in an interferon regulatory factor 3 (IRF-3)-mediated, but IFN-independent way (Grandvaux et al., 2005).

Thus, it is known from literature that ARG II can be induced by several agents, like dibutyryl-cAMP, PGE_2, LPS, IRF-3, LXR, IL-10/isoproterenol or AC-derived mediators, but the underlying mechanisms still have to be identified and characterized.

3.7 Aims of the studies

As professional phagocytes, MΦs engulf ACs to prevent them from undergoing secondary necrosis, thereby protecting the surrounding tissue from being exposed to inflammatory constituents. Moreover, as mentioned before, ACs polarize MΦs towards a regulatory phenotype (M2c) (Mosser and Edwards, 2008), characterized by enhanced production of anti-inflammatory IL-10, TGF-β1 and attenuated pro-inflammatory mediators, like NO. Recently, Johann *et al.* demonstrated that ARG II up-regulation in response to ACs or AC-derived mediators attenuated NO production in RAW264.7 MΦs (Johann et al., 2007). ARG II competes with iNOS for the common substrate L-arginine thereby impairing NO formation (Morris, 2004). These findings corroborated other studies reporting modulation of NO production in MΦs by ARG II (Gotoh and Mori, 1999; Topal et al., 2006) (see 3.4.2). Based on these observations, ARG II seems to be a promising therapeutical target in pathophysiological settings derived from detrimental NO adjustment and thus, understanding molecular pathways of its regulation is necessary. Surprisingly, despite the important contribution of ARG II in the regulation of NO production in MΦs, the mechanism mediating ARG II up-regulation has not been identified up to date.

Considering the rudimentary information on such a pathway, I intended to characterize the signaling cascade used by AC-derived mediators to up-regulate ARG II in MΦs. Furthermore, since the intracellular pathways responsible for the regulatory MΦ phenotype remain elusive and ARG expression is an important hallmark of this type of MΦs, I speculated that the elucidation of the ARG II-inducing mechanism would provide additional information on AC-dependent MΦ phenotype switch.

4 Materials and Methods

4.1 Materials

4.1.1 Cells

RAW 264.7 cells:
RAW 264.7 mouse monocytes/macrophages were established from ascites of a tumor induced in a male Balb/c mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV) in 1978 (Raschke et al., 1978).

Jurkat T cells:
Jurkat T cells are derived from a 14 year old boy with acute lymphatic leukemia (ALL). This T cell line was established in 1977 (Schneider and Schwenk, 1977).

MCF-7 cells:
MCF-7 human breast adenocarcinoma cells were derived from the pleural effusion of a 69-year-old Caucasian woman with metastatic mammary carcinoma in 1970 (Soule et al., 1973).

HEK 293 cells:
HEK 293 cells were generated by transformation of human embryonic kidney cells with sheared adenovirus 5 DNA. This cell line was first described in 1977 (Graham et al., 1977).

Primary murine macrophages:
Primary peritoneal macrophages were isolated by peritoneal lavage from C57BL/6 mice. All procedures performed on these mice followed the guidelines of the Hessian animal care and use committee.

4.1.2 Bacteria

Vectors were amplified in XL1-Blue® supercompetent bacteria, except the pcDNA3-FLAG1-MEK5 WT plasmid obtained by QuikChange® II XL site directed mutagenesis, whose amplification occurred in XL10-Gold®

ultracompetent cells. Bacteria were provided by Stratagene GmbH (Amsterdam, The Netherlands) and are listed in Table 4.1.

Table 4.1: Bacteria strains

Bacteria strain	Genotype
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZ.M15 Tn10(Tetr)].
XL10-Gold	TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB lacIqZDM15 Tn10 (Tetr) Amy Camr].

4.1.3 Chemicals and reagents

All chemicals were of highest grade of purity and commercially available. Cell culture media and supplements came from PAA (Cölbe). Reagents and kits are listed in Table 4.2.

Table 4.2: Reagents and kits

Chemical/Kit	Provider
AACOCF$_3$ (phospholipase A_2 inhibitor)	Calbiochem (Darmstadt, Germany)
Allstars negative control siRNA	Qiagen GmbH (Hilden, Germany)
Amaxa® Nucleofector® Kit V	Lonza Cologne AG (Köln, Germany)
Direct Cyclic AMP EIA Kit	Assay Designs (Ann Arbor, MI, USA)
Cellulose syringe filter (0.2-µm)	Roth (Karlsruhe, Germany)
D-luciferine	AppliChem GmbH (Darmstadt, Germany)
Forskolin (adenyly cyclase activator)	Alexis Biochemicals (Lörrach, Germany)
GW4896 (sphingomyelinase inhibitor)	Calbiochem (Darmstadt, Germany)
HiSpeed Plasmid Maxi Kit	Qiagen GmbH (Hilden, Germany)
IBMX (nonselective phosphodiesterase inhibitor)	Biomol GmbH (Hamburg, Germany)
In-Fusion™ Dry-Down PCR Cloning Kit	Clontech-Takara (Saint-Germain-en-Laye, France)
Janus kinase inhibitor 1	Calbiochem (Darmstadt, Germany)
JetPEI™ transfection reagent	Polyplus transfection (Illkirch, France)

Chemical/Kit	Provider
JTE013 (sphingosine-1-phosphate receptor 2 antagonist)	Cayman Chemical (Ann Arbor, MI, USA)
KT5720 (protein kinase A inhibitor)	Biomol GmbH (Hamburg, Germany)
Luminol (3-Aminophtalhydrazide)	Acros Organics (Geel, Belgium)
LY294002 (phosphatidylinositol 3-kinase inhibitor)	Alexis Biochemicals (Lörrach, Germany)
N-oleoyl-ethanolamine (ceramidase inhibitor)	Calbiochem (Darmstadt, Germany)
ON-TARGETplus SMARTpool siRNA (murine extracellular signal-regulated kinase 5 and sphingosine-1-phosphate receptor 2)	Dharmacon RNAi Technologies (Bonn, Germany)
PD98059 (ERK1/2 inhibitor)	Alexis Biochemicals (Lörrach, Germany)
Proteinase K	New England Biolabs (Frankfurt, Germany)
QuikChange® XL II site directed mutagenesis kit	Stratagene (Amsterdam, The Netherlands)
Restriction enzymes (*Bgl II, EcoR V, Hind III* and *Sma I*)	New England Biolabs (Frankfurt, Germany)
Rolipram (phosphodiesterase 4D inhibitor)	Biomol GmbH (Hamburg, Germany)
SB203580 (p38 inhibitor)	Sigma-Aldrich GmbH (Deisenhofen, Germany)
siControl nontargeting siRNA	Dharmacon RNAi Technologies (Bonn, Germany)
Sphingosine-1-phosphate	Avanti (Alabaster, AL, USA)
SQ22536 (adenyly cyclase inhibitor)	Biomol GmbH (Hamburg, Germany)
Staurosporine	Sigma-Aldrich GmbH (Deisenhofen, Germany)
U0126 (MEK1/2/5 inhibitor)	Alexis Biochemicals (Lörrach, Germany)
U73122 (phospholipase C inhibitor)	Sigma-Aldrich GmbH (Deisenhofen, Germany)

4.1.4 Antibodies

IRDye680 or 800-labelled secondary antibodies from mouse and rabbit were obtained from Li-COR Biosciences GmbH (Bad Homburg, Germany) (dilution for WB analysis 1:6000). Primary antibodies are listed in Table 4.3.

Table 4.3: Primary antibodies

Antibody	Provider	Dil. for WB
Anti-tubulin	Sigma-Aldrich GmbH (Deisenhofen, Germany)	1:2000
Anti-actin	Sigma-Aldrich GmbH (Deisenhofen, Germany)	1:2000
Anti-ARG II	Santa Cruz Biotechnology, Inc. (Heidelberg, Germany)	1:1000
Anti-CREB-1 (100x)	Santa Cruz Biotechnology, Inc. (Heidelberg, Germany)	1:25
Anti-S1P$_2$ (EDG-5)	Santa Cruz Biotechnology, Inc. (Heidelberg, Germany)	1:1000
Anti-ERK1/2	Cell Signaling (Danvers, MA, USA)	1:1000
Anti-ERK5	Cell Signaling (Danvers, MA, USA)	1:1000
Anti-HA.11	Covance (Emeryville, CA, USA)	1:1000
Anti-FLAG	Rockland (Gilbertsville, PA, USA)	1:1000

4.1.5 Plasmids

Plasmids used in this study are listed in the tables below (Table 4.4 and Table 4.5). The murine ARG II promoter (mpARG II) was cloned into pGL3-Basic as described (Johann et al., 2007) in order to follow mpARG II activity.

Table 4.4: Reporter plasmids

Plasmid	Informations on properties	Generation
pGL3-Basic	no promoter upstream of the luciferase encoding region, ampicillin resistance	Promega (Mannheim, Germany)
pGL3-mpARG II	mpARG II (1856 bp), no promoter upstream of the luciferase encoding region, ampicillin resistance	(Johann et al., 2007)
pGL3-mpARG II-1359 bp	deletion construct of mpARG II (1359 bp), no promoter upstream of the luciferase encoding region, ampicillin resistance	Double digest of pGL3-mpARG II (**EcoR V**, **Bgl II**)

Plasmid	Informations on properties	Generation
pGL3-mpARG II-442 bp	deletion construct of mpARG II (442 bp), no promoter upstream of the luciferase encoding region, ampicillin resistance	Double digest of pGL3-mpARG II (**EcoR V**, **Hind III**)
pGL3-mpARG II-262 bp	deletion construct of mpARG II (262 bp), no promoter upstream of the luciferase encoding region, ampicillin resistance	Double digest of pGL3-mpARG II (**EcoR V**, **Sma I**)

Table 4.5: Over-expression plasmids

Plasmid	Description of encoded protein	Provider/Production
pcDNA3-HA1-ERK5N	HA1-tagged truncated form of ERK5 (residues 1-407) containing the TEY activation motif	Prof. Eisuke Nishida (Kyoto University, Japan)
pcDNA3-FLAG1-MEK5D	FLAG1-tagged constitutive active form of MEK5, where serine 311 and threonine 315 were replaced by aspartate	
pcDNA3-FLAG1-MEK5 WT	FLAG1-tagged wild type form of MEK5	QuikChange point-mutation of pcDNA3-FLAG1-MEKD

4.1.6 Oligonucleotides

Oligonucleotides were bought from Biomers.net GmbH (Ulm, Germany) and are listed in Table 4.6 in 5'-3' sence. Boldface letters in EMSA and decoy-oligonucleotides indicate the consensus sequence of the CRE-site. The underlined letters represent the mutated bases (AC → TG). Point mutations in QuikChange primers (boldface letters) were used to replace aspartate by serine 311 and threonine 315 in pcDNA3-FLAG1-MEKD in order to generate an over-expression plasmid encoding the FLAG1-tagged wild type form of MEK5.

Table 4.6: Oligonucleotides

Primer	5'-forward-3'	5'-reverse-3'
mpARG II-CRE (EMSA) IRD700-labeled or unlabeled	GAG AGA TGC **TGA CGT C A**C AGG GCG GTG	CAC CGC CCT G**TG ACG TCA** GCA TCT CTC

Primer	5'-forward-3'	5'-reverse-3'
mpARG II-CRE mut (EMSA) unlabeled	GAG AGA TGC **TGT GGT C A**C AGG GCG GTG	CAC CGC CCT G**TG ACC ACA** GCA TCT CTC
mpARG II-CRE-decoy (decoy approach) fluorescein-labeled	GAG AGA TGC **TGA CGT C A**C AGG GCG GTG	CAC CGC CCT G**TG ACG TCA** GCA TCT CTC
mpARG II-CRE-decoy mut. (decoy approach) fluorescein-labeled	GAG AGA TGC **TGT GGT C A**C AGG GCG GTG	CAC CGC CCT G**TG ACC ACA** GCA TCT CTC
MEK5 WT (QuikChange)	GGC GTG AGC ACA CAG C TG GTG AAT **TC**T ATA GC C AAG **ACG** TAT GTT GGA AC	GT TCC AAC ATA **CGT** CTT GGC TA T A**GA** ATT CAC CAG CTG TGT GCT CAC GCC

4.1.7 Instruments and Software

Instruments and software used in this study are listed in Table 4.7 and Table 4.8.

Table 4.7: Instruments

Instruments	Provider
Autoclave HV 85	BPW GmbH (Süssen, Germany)
B250 Sonifier	Branson Ultrasonics (Danbury, USA)
Bacteria clean bench UV/UB 1200	Uniequip GmbH (Martinsried, Germany)
Bacteria incubator B5042	Heraeus GmbH (Hanau, Germany)
CASY®	Schärfe System (Reutlingen, Germany)
Centrifuge 5415 R	Eppendorf GmbH (Hamburg, Germany)
Centrifuge CR 3.22 and MR23i	Jouan GmbH (Unterhaching, Germany)
FACSCanto flow cytometer	BD Biosciences GmbH (Heidelberg, Germany)
Fluorescence microscope Axiovert 200M	Carl Zeiss MicroImaging Inc (Göttingen, Germany)
Holten Lamin Air clean bench	Jouan GmbH (Unterhaching, Germany)

Instruments	Provider
IG 150 (cell culture incubator)	Jouan GmbH (Unterhaching, Germany)
LabLine Orbit Shaker	Uniequip GmbH (Martinsried, Germany)
Magnetic stirrer Combimag RCH	IKA Labortechnik GmbH & Co. KG (Staufen, Germany)
Mastercycler®	Eppendorf GmbH (Hamburg, Germany)
Mini-PROTEAN 3 System	Bio-Rad Laboratories GmbH (Munich, Germany)
Mithras LB940 multimode reader	Berthold Technologies (Bad Wildbad, Germany)
NanoDrop ND-1000	Peqlab Biotechnologie GmbH (Erlangen, Germany)
Nucleofector	Amaxa AG (Cologne, Germany)
Odyssey infrared imaging system	Li-COR Biosciences GmbH (Bad Homburg, Germany)
Pure water system Purelab Plus	ELGA LabWater GmbH (Siershahn, Germany)
Sub-Cell® GT electrophoresis system	Bio-Rad Laboratories GmbH (Munich, Germany)
Thermomixer 5436	Eppendorf GmbH (Hamburg, Germany)
Trans-Blot SD blotting machine	Bio-Rad Laboratories GmbH (Munich, Germany)
Ultrasonic bath Sonorex	Bandelin electronic GmbH (Berlin, Germany)
UV-Transilluminator gel documentation system	Raytest GmbH (Straubenhardt, Germany)

Table 4.8: Software

Software	Provider
AxioVision Software	Carl Zeiss MicroImaging Inc (Göttingen, Germany)
BD FACSDivaTM Software	BD Biosciences GmbH (Heidelberg, Germany)
MikroWin 2000 Software	Berthold Technologies (Bad Wildbad, Germany)
Odyssey 2.1 Software	Li-COR Biosciences GmbH (Bad Homburg, Germany)

4.2 Methods

4.2.1 Cell biology

4.2.1.1 Cell culture

Human Jurkat T cells, RAW264.7 mouse MΦs, human MCF-7 breast carcinoma cells and primary murine peritoneal MΦs were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium. Human embryonic kidney (HEK 293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose. Media were supplemented with 10% heat-inactivated FCS, 5 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were kept in a humidified atmosphere of 5% CO_2 at 37℃ and transferred twice a week. Cells were regularly tested to be free of mycoplasma. Cell numbers were determined using the cell counter system Casy®.

4.2.1.2 Isolation and culture of murine peritoneal macrophages

Murine peritoneal MΦs were harvested by rinsing the peritoneal cavity with icecold PBS. MΦs were purified by adherence to tissue culture plastic and three subsequent washing steps, followed by resting for 3 days in RPMI 1640 supplemented with 10% heat-inactivated FCS, non-essential amino acids, pyruvate, 5 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin before individual stimulation.

4.2.1.3 Generation of apoptotic Jurkat and MCF-7 cells

MCF-7 or Jurkat cells were treated for 2.5 hours with 0.5 µg/ml staurosporine in FCS free RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, washed three times with medium to remove staurosporine and incubated for another 5 hours in full medium to generate apoptotic cell (AC)-conditioned medium (CM) ($2.5 * 10^6$ ACs/ml medium). Cell death (~80%) was confirmed by flow cytometry, using annexin V and propidium iodide staining (Immunotech, Marseille, France). ACs were centrifuged for 10 minutes at 1000 x g to collect the medium. The supernatant of ACs was passed through a 0.2-µm cellulose syringe filter (Roth, Karlsruhe, Germany) to remove remaining cells and debris. The filtrated supernatant was taken as CM. I used both CM from apoptotic MCF-7 (CM-M) and Jurkat cells (CM-J), since CM from different

ACs show similar effects on MΦs (Weigert et al., 2006; Weigert et al., 2007). In all experiments, the concentration of CM-M corresponded to the ratio of 1 MΦ to 2 ACs (ratio 1MΦ:2ACs), while CM-J was used in a ratio of 1 MΦ to 5 ACs (ratio 1MΦ:5ACs).

4.2.1.4 Generation of modified conditioned media

Jurkat cells were pre-incubated with the sphingomyelinase inhibitor GW4896, the ceramidase inhibitor n-oleoyl-ethanolamine (NOE), the phospholipase A_2 (PLA_2) inhibitor $AACOCF_3$ or the phospholipase C (PLC) inhibitor U73122 for 30 minutes, followed by induction of apoptosis as described. In order to digest and denaturate proteins, CM was incubated with 50 µg/ml proteinase K at 37℃ for 1 hour, followed by incubation at 100℃ for 1 h our.

4.2.1.5 Generation of a stable sphingosine kinase 2 knock-down in MCF-7 cells

For sphingosine kinase 2 (SK2) knock-down, pSilencer-siSK2 was stably transfected into MCF-7 cells (MCF-7-siSK2) using Nucleofector technology (Amaxa, Köln, Germany) as described (Weigert et al., 2007). MCF-7-Neo cells were generated by nucleofection of MCF-7 cells with the pSilencer 4.1-CMV neo control vector (Ambion, Darmstadt, Germany).

4.2.1.6 Transient transfection

Transient transfection of different over-expression vectors and reporter plasmids was performed using JetPEI™ cationic polymer transfection reagent according to the instructions of the manufacturer.

For reporter analysis $5 * 10^5$ RAW264.7 cells were seeded per well in 24-well plates. Cells were allowed to adhere for 4 hours followed by transfection. In brief, 1 µg DNA and 2 µl JetPEI™ transfection reagent were mixed each with 25 µl 150 mM NaCl per well and vortexed briefly. The JetPEI™ mixture was added to the DNA mixture, vortexed, spinned down and incubated for 30 minutes at room temperature (RT). Afterwards 50 µl/well of the solution were added to the cells, which incubated for 4 hours before changing culture medium. Incubations continued for 24 hours, followed by individual stimulation. Luciferase activity was measured 15 hours later.

For over-expression experiments 8 * 10^5 HEK 293 cells were seeded in 10 cm dishes. The next day, cells were transfected with pcDNA3-HA1-ERK5N in combination with pcDNA3-FLAG1-MEK5 WT or pcDNA3-FLAG1-MEK5D. In brief, 10 µg DNA (5 µg of each plasmid) and 20 µl JetPEI™ transfection reagent were mixed each with 250 µl 150 mM NaCl per dish and vortexed briefly. The JetPEI™ mixture was added to the DNA mixture, vortexed, spinned down and incubated for 30 minutes at RT. Afterwards 500 µl/dish of the solution were added to the cells, which incubated for 4 hours before starving overnight, followed by individual stimulation.

Transient transfection of siRNA was performed using the Nucleofector® technology from Amaxa biosystems. According to the manufacturer's protocol, 2 * 10^6 RAW264.7 MΦs were centrifuged for 10 minutes at 90 x g and re-suspended in 100 µl Nucleofector® Solution V. After addition of 3 µg siRNA cells were nucleofected using program "D-032". Transfected cells were immediately transferred in 3 ml pre-warmed medium and seeded on 6 cm plates. Culture medium was changed after 4 hours. Experiments were done 48 hours after transfection.

4.2.1.7 CREB decoy experiments

The decoy approach is based on the ability of living cells to incorporate double-stranded, phosphothiorate stabilized oligonucleotides, which contain a consensus binding sequence for the transcription factor of interest. These decoy-oligonucleotides scavenge the transcription factor by occupying the DNA-binding site, thereby preventing it from binding to promoter regions of target genes (von Knethen et al., 1998). RAW264.7 cells were transiently transfected with CRE-oligonucleotides containing the CRE-consensus site derived from mpARG II and oligonucleotides with a mutated site as a control (see Table 4.6). In addition, CREB decoy-oligonucleotides contained a 5'-terminal fluorescein label. One day before transfection cells were seeded at a density of 1 * 10^6 cells in 6 cm dishes. Oligonucleotides (3 µM) were added 24 hours prior to experiments. After changing the medium, individual stimulations were performed. Transfection efficiency was determined by counting labeled cells by fluorescence-microscopy and/or FACS analysis.

4.2.2 Biochemistry

4.2.2.1 Protein determination (Lowry)

The protein content of cell lysates was determined by using the DC Protein Assay Kit, based on the Lowry method (Lowry et al., 1951). Briefly, a standard dilution series of BSA in protein lysis buffer (see Appendix I) was prepared (0.625 to 10 mg/ml). 2 µl of the the standard dilution series as well as of samples were pipetted in duplicates into a 96-well plate, 20 µl solution A were added, and then the colorimetric reaction was started by addition of 160 µl solution B. After incubation for 15 minutes (RT, shaking), extinction was measured at 750 nm using the Mithras LB 940 multimode reader.

4.2.2.2 Nuclear protein extraction

$3 * 10^6$ RAW264.7 MΦs were seeded in 10 cm dishes and stimulated with CM for 6 respectively 16 hours with or without pharmacological inhibitors. Cells were washed with ice-cold PBS and lysed with 200 µl ice cold hypotonic cell lysis buffer (see Appendices) for 15 minutes on ice. Nuclei were sedimented by centrifugation at 12000 x g for 1 minute and cytosolic fraction was transferred in a new tube. Subsequently, sedimented nuclei were lysed with 50 µl nuclear lysis buffer (see Appendix I), incubated for 30 minutes on ice and centrifuged at 12000 x g for 10 minutes. Supernatant was transferred in a new tube and protein content was determined using the Lowry method (see 4.2.2.1)

4.2.2.3 SDS-PAGE/Western analysis

For Western analysis, $1\text{-}3 * 10^6$ HEK 293 cells, RAW264.7 or primary peritoneal MΦs were stimulated as indicated, scraped off, lysed with 200 µl protein lysis buffer (see Appendix I) and sonicated for 10 impulses. Then, lysates were incubated on ice for 30 minutes and vortexed every 5 minutes. After centrifugation (4°C, 16000 x g, 30 minutes) supernatants were transferred into a new tube. Protein concentrations were determined as described above (see 4.2.2.1). 60-100 µg from RAW264.7 or HEK 293 cells, or 30 µg of protein from primary peritoneal MΦs were mixed with 4 x SDS sample buffer and denaturated for 10 minutes at 95°C. Proteins were separated on 10% SDS-polyacrylamide geles using 1 x SDS-running buffer and the Mini-PROTEAN 3 system (see Appendix I). Proteins were transferred onto a nitrocellulose

membrane by semi-dry blotting. To prevent unspecific binding, membranes were blocked with 5% BSA/TTBS (see Appendix I) for 2 hours at RT. Afterwards membranes were incubated with antibodies in 5% BSA/TPBS at 4℃ over night at indicated concentrations (see Table 4.3). For protein detection, membrane was washed 3 times with TTBS for 7 minutes and incubated with IRDye secondary antibodies (anti-rabbit or anti-mouse; 1:6000) in 5% BSA/TTBS for 1 hour at RT. After washing the membrane 3 times for 7 minutes proteins were visualized and densitometrically analyzed with the Odyssey infrared imaging system.

4.2.3 Molecular biology

4.2.3.1 Reporter assay

All reporter assays were performed in duplicate. For reporter analysis cells were re-suspended in 100 µl reporter lysis buffer (see Appendix I) and incubated for 15 minutes under shaking at RT. Lysates were cleared by 4000 x g centrifugation for 5 minutes. Luciferase activity was measured using a Mithras LB 940 multimode reader. For this purpose, 20 µl of the lysates were transferred into a 96-well plate. 50 µl reporter assay reagent were added automatically, the plate shaked for 2 seconds and each well was measured for 10 seconds. Luciferase activity was normalized to protein concentration of each sample.

4.2.3.2 Electrophoretic mobility shift assay (EMSA)

Except some modifications, EMSA was performed as previously described (Von Knethen and Brune, 2001; Weigert et al., 2007). 10 µg nuclear protein (see 4.2.2.2) were incubated with 2 µg poly(dIdC), 2 µl buffer D, 4 µl buffer F (see Appendix I), and 250 fmol 5'-IRDye700-labeled mpARG II-CRE oligonucleotides (see Table 4.6) in a final volume of 20 µl and incubated for 20 minutes at RT. Supershift analysis was performed by adding 4 µg of anti-CREB-1 for 30 minutes prior to incubation with 5'-IRDye700-labeled oligonucleotides for 20 minutes. The competition assay was performed by adding 25, 50 or 100 fold unlabeled mpARG II-CRE or mpARG II-CRE mut oligonucleotides (see Table 4.6). DNA-protein complexes were resolved on native 4% polyacrylamide gels with 200 V for approximately 1 hour. The intensity of the bands, corresponding

to specific CREB-DNA binding, was determined using the Odyssey infrared imaging system.

4.2.3.3 Enzyme immunoassay (EIA)

Determination of cAMP levels in RAW264.7 MΦs was performed by a Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs) according to the manufacturer's instructions. $3 * 10^6$ RAW264.7 MΦs were seeded in 10 cm dishes and starved over night prior to individual stimulations. Cells were lysed with 300 µl 0.1 M HCl.

4.2.3.4 Construction of the pGL3-mpARG II deletion constructs

An ARG II luciferase reporter plasmid (pGL3-Basic-mpARG II) containing mpARG II upstream of the luciferase gene was generated as described (Johann et al., 2007) to follow ARG II promoter activity. Promoter deletion constructs of pGL3-Basic-mpARG II were produced in order to narrow the region of mpARG II responsible for CM-mediated ARG II up-regulation down. Therefore 1 µg of pGL3-Basic-mpARG II plasmid was double-digested with restriction enzymes as listed in Table 4.9. The reaction mixture was filled up to 25 µl with double-distilled, steril H_2O.

Table 4.9: Double-digestion of plasmids.

Enzymes	**NEBuffer + 2.5 µg BSA**	**Restriction temperature, time**	**Klenow reaction**
EcoR V (0.5 µl); Bgl II (0,5 µl)	Buffer 3	37℃, 1.5 hours	yes
EcoR V (0.75 µl); Hind III (0.5 µl)	Buffer 2	37℃, 1.5 hours	yes
EcoR V (1 µl); Sma I (0.5 µl)	Buffer 4	25℃, 37℃, each 1,5 hours	no

After double-digest, the restriction enzymes were inactivated by heat (80℃, 20 minutes). Bgl II and Hind III endonucleases produce 5'-overhanging ends, which have to be filled to blunt ends by Klenow reaction in order to be able to re-ligate the linearized vectors. The Klenow reaction is achieved at 30℃ for 15 minutes, followed by inactivation at 70℃ for 10 minutes (Table 4.10).

Table 4.10: Klenow reaction.

Double-digest reaction	10 µl
10 x Klenow reaction buffer	2 µl
dNTPs	2 µl
Klenow DNA polymerase (1 U/µl)	1 µl
ddH_2O ad 20 µl	5 µl

The linearized vectors were re-ligated by T4 ligase at 22℃ for 1 hour followed by inactivation for 20 minutes at 65℃.

Table 4.11: Ligation of linearized vectors.

Klenow reaction respectively Double-digest	4 µl respectively 2 µl
10 x Ligation buffer	2 µl
dNTPs	2 µl
T4 ligase	5 µl
ddH_2O ad 20 µl	7 µl respectively 9 µl

Double-digest with EcoR V in combination with Bgl II, Hind III or Sma I produced a -1359 bp to -1 bp, a -442 bp to -1 bp or a -262 bp to -1 bp construct of mpARG II (FIGURE 4.1). Successful cloning of the deletion constructs was verified by sequencing (Agowa GmbH, Berlin, Germany).

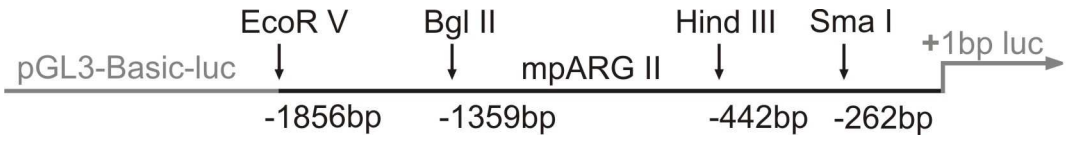

FIGURE 4.1: Restriction enzymes used to generate mpARG II promoter constructs.

4.2.3.5 Site directed mutagenesis for generation of point mutations

In order to obtain an over-expression plasmid encoding for the wild type form of MEK5, site directed mutagenesis of pcDNA3-FLAG1-MEK5D was performed using QuikChange® II XL site directed mutagenesis kit. Primers were designed according to manufacturer's instructions and are listed in Table 4.6. 5 µl

10 x reaction buffer, 10 ng of the double-stranded DNA template, 125 ng of each primer, 1 µl dNTP mix, 3 µl QuikSolution were mixed and filled up with distilled H_2O to a final volume of 50 µl. Then 1 µl PfuUltra HF DNA polymerase (2.5 U/µl) were added. The reaction was performed with the cycling parameters outlined in Table 4.12. After temperature cycling, the reaction tubes were placed on ice for 2 minutes to cool the reactions to $\leq$ 37℃, followed by the digestion of non-mutated parental DNA template with 10 U *DpnI* for 2 hours at 37℃. Mutated plasmids were transformed into XL10-Gold® Ultracompetent bacteria using the heat shock method (see 4.2.4.1). Plasmids from potential positive clones were isolated and verified by sequencing (Agowa GmbH, Berlin, Germany).

Table 4.12: Cycling parameters for QuikChange® II XL method

Cycles	Segment	Temperature	Time
1	initial denaturation	95℃	1 minute
18	denaturation	95℃	50 seconds
	anneling	60℃	50 seconds
	extension	68℃	7 minutes
1	final extension	68℃	10 minutes

4.2.4 Microbiology

4.2.4.1 Transformation of bacteria by heat-shock

Bacteria were transformed with plasmid DNA by heat-shock. Therefore, 45 µl of bacteria glycerol stocks were thawed on ice and 2 µl of β-mercaptoethanol were added in order to increase transformation efficiency. After incubation on ice for 10 minutes and swirling every 2 minutes, 50 ng plasmid DNA were added, followed by another 30 minutes on ice. After heat-shock (40 seconds, 42℃), bacteria were incubated for another 2 minutes on ice. For initial growth, 500 µl of pre-heated SOC medium (see Appendices) were added followed by an incubation period of 1 hour at 37℃ with shaking at 250 rpm. Then, 200 µl were inoculated on a LB agar plate containing 100 µg/ml ampicillin and incubated over night at 37℃ to select positive, plasmid-carr ying bacteria clones.

4.2.4.2 Bacterial culture and plasmid preparation

For preparation of plasmids a single clone from the LB agar plate was picked, transferred into 3 ml LB medium containing 100 µg/ml ampicillin and cultured over night at 37℃ with shaking (250 rpm). The next day, the culture was transferred into 200 ml LB medium containing the appropriate antibiotic and again shaked over night at 37℃. Isolation of plasm ids was performed according to the manufacturer's protocol using the NucleoSpin® Plasmid Kit. DNA content was measured with the Nanodrop ND-1000.

4.2.5 Statistical analysis

Data represented in graphs are means ± SEM of at least three independent experiments. Statistical analysis was done using the paired Student's t-test and considered significant (*) at $p \leq 0.05$, (**) at $p \leq 0.005$ and (***) at $p \leq 0.0005$. Western blots displayed in this study are representative of at least three independent experiments.

5 Results

Johann *et al.* demonstrated that apoptotic Jurkat cells induced ARG II protein expression in MΦs, thereby modulating NO production. Cell-cell contact was not necessary since supernatants of ACs, termed CM, showed the same effect. The authors concluded that ACs secrete soluble factors responsible for ARG II up-regulation (Johann et al., 2007). I noticed that supernatants from apoptotic MCF-7 cells also induced ARG II, implicating that its expression is independent of the AC type being used. A detailed signaling pathway mediating ARG II up-regulation in MΦs was still missing. Since ARG expression is a hallmark of the regulatory MΦ phenotype (Mantovani et al., 2004b), I assumed that elucidation of the underlying pathway(s) could provide general information on intracellular mechanisms responsible for the development and maintenance of this phenotype.

5.1 Sphingosine-1-phosphate (S1P) from apoptotic cells (ACs) activates S1P receptor 2 ($S1P_2$) to up-regulate arginase II (ARG II) in macrophages (MΦs)

5.1.1 ACs secrete soluble lipid factors that up-regulate ARG II expression

To provide information on principal pathways up-regulating ARG II, I first time-dependently determined ARG II expression in response to apoptotic MCF-7 cell-conditioned medium (CM-M) in RAW264.7 MΦs. ARG II protein expression was already detectable after 4 hours, followed by a steady increase up to 30-fold induction after 20 hours of incubation (FIGURE 5.1 A). These data correlate with observations made by Johann *et al.*, who showed a strong ARG II protein expression in RAW264.7 MΦs after 24 hours following addition of apoptotic Jurkat cell CM (CM-J) (Johann et al., 2007).

Having established my experimental setup, I was interested in the characterization of the soluble factor(s) in CM mediating ARG II up-regulation. In order to analyze the role of proteins in CM-M, I digested them with 50 µg/ml proteinase K (at 37°C for 1 hour) followed by denaturation for 1 hour at 100°C. This CM-M, lacking functional proteins, still showed ARG II induction in MΦs

(FIGURE 5.1 B), excluding AC-derived proteins such as TGF-β, IL-4 and IL-10, as the responsible factors.

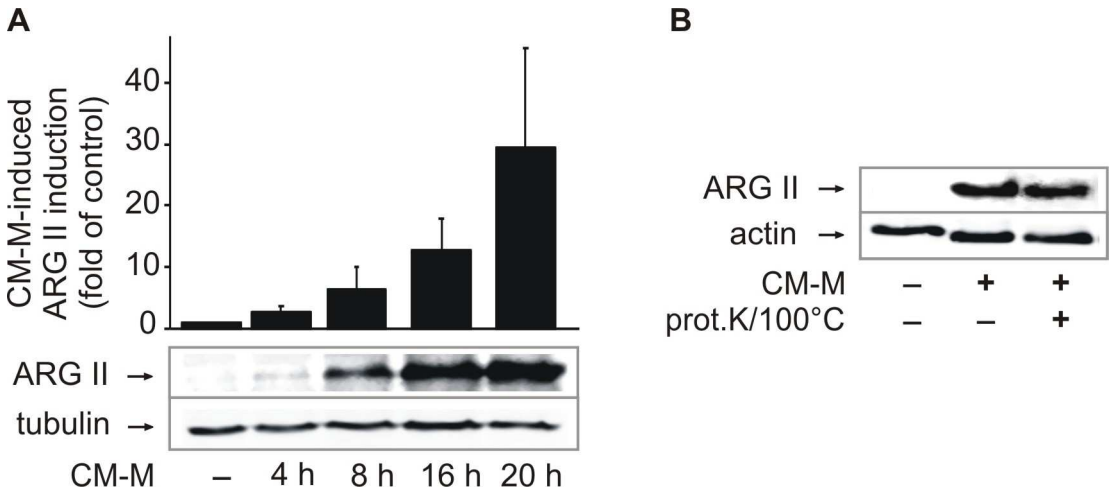

FIGURE 5.1: CM-M up-regulates ARG II in MΦs in a protein-independent way.
(A) MΦs were incubated for the indicated times with CM-M. The graph shows quantification of CM-M-mediated ARG II protein expression normalized to tubulin. ARG II expression in RAW264.7 cells was determined by Western analysis. (B) Cells were incubated for 16 hours with CM-M or CM-M treated with 50 µg/ml proteinase K (prot.K) at 37℃ for 1 hour, followed by 1 hour at 100℃ in order to digest and denaturate proteins. ARG II expression in RAW264.7 cells was determined by Western analysis.

5.1.2 Lipid factors in apoptotic cell-conditioned medium (CM) up-regulate ARG II

After excluding AC-derived proteins, I concentrated on the possibility of soluble lipid factors as the responsible mediators. Taking into account that glycerophospholipid as well as sphingolipid metabolism are active in ACs and their metabolites are involved in differentiation, immunity and survival of MΦs (Goetzl et al., 2004; Lauber et al., 2003; Weigert et al., 2006), I considered the possibility that they might be involved in ARG II expression. Therefore, I inhibited the enzymes of glycerophospholipid metabolism PLA_2 using $AACOCF_3$ and PLC using U73122 (FIGURE 5.2 A,B) before inducing apoptosis in Jurkat cells. PLA_2 hydrolizes phosphatidylcholine (PtdCh) to LPC and arachidonic acid (AA) subsequently generating PGs, while PLC produces inositol 1,4,5-trisphophate (IP_3), diacylglycerol (DAG) and down-stream lysophosphatidic acid (LPA) through phosphatidylinositol (PtdIns) hydrolysis. Thus, these modified conditioned media CM-J_{AACOCF_3} and CM-J_{U73122} should have reduced amounts

of PLA_2 respectively PLC products. I exposed RAW264.7 MΦs to CM-J_{AACOCF_3} and CM-J_{U73122}, determined ARG II protein expression and observed no alteration in comparison to CM-J-mediated ARG II up-regulation (FIGURE 5.2 C). These data suggest that AC-derived glycerophospholipids LPC, LPA and the second messenger IP_3 as well as PGs do not contribute to ARG II up-regulation in MΦs.

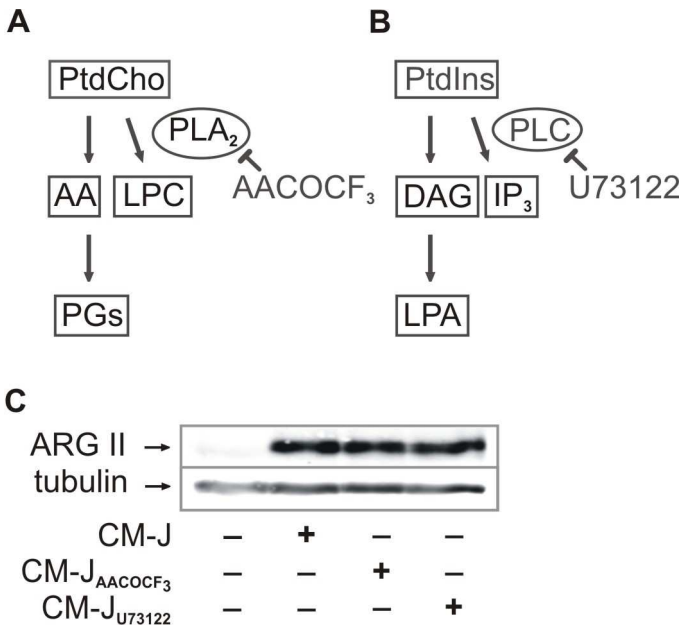

FIGURE 5.2: Contribution of glycerophospholipids to ARG II induction in MΦs. (A,B) Simplified schemes of glycerophospholipid metabolisms: Enzyme inhibitors used in this study are indicated. (PtdCh: phosphatidylcholine, PtdIns: phosphatidylinositol, AA: arachidonic acid, LPC: lysophosphatidylcholine, PG: prostaglandins, DAG: diacylglycerol, IP3: inositol 1,4,5-trisphosphate, LPA: lysophosphatidic acid). (C) Cells were incubated for 16 hours with CM-J or modified conditioned media from Jurkat cells where PLA_2 or PLC had been inhibited with AACOCF3 or U73122. ARG II expression in RAW264.7 cells was determined by Western analysis.

On the contrary, consecutive inhibition of the sphingolipid metabolizing enzymes (FIGURE 5.3 A) before inducing apoptosis in Jurkat or MCF-7 cells, generated modified conditioned media, which were less active in ARG II up-regulation (FIGURE 5.3 B,C). In fact, the SMase inhibitor GW4869 or the CDase inhibitor NOE in apoptotic Jurkat cells reduced the potential of CM-Js to up-regulate ARG II (FIGURE 5.3 B). These data pointed to the importance of the sphingolipid mediator S1P for ARG II expression, since ceramide and sphingosine, produced by SMase and CDase respectively, are its precursors. Weigert *et al.* previously generated a stable SK2 knock-down MCF-7 cell-line,

whose CM (CM-M_{siSK2}) showed a reduced S1P content compared to CM-M_{Neo} (4 ng/ml S1P vs. 0.65 ng/ml S1P in the supernatants of 2 * 10^5 apoptotic MCF-7-Neo vs. MCF-7-siSK2 cells) (Weigert et al., 2007). I used CM-M_{siSK2} to demonstrate the requirement of S1P for ARG II induction. As expected, CM-M_{siSK2} induced less ARG II expression compared to CM-M_{Neo} (FIGURE 5.3 C), thus corroborating the role of S1P for ARG II up-regulation.

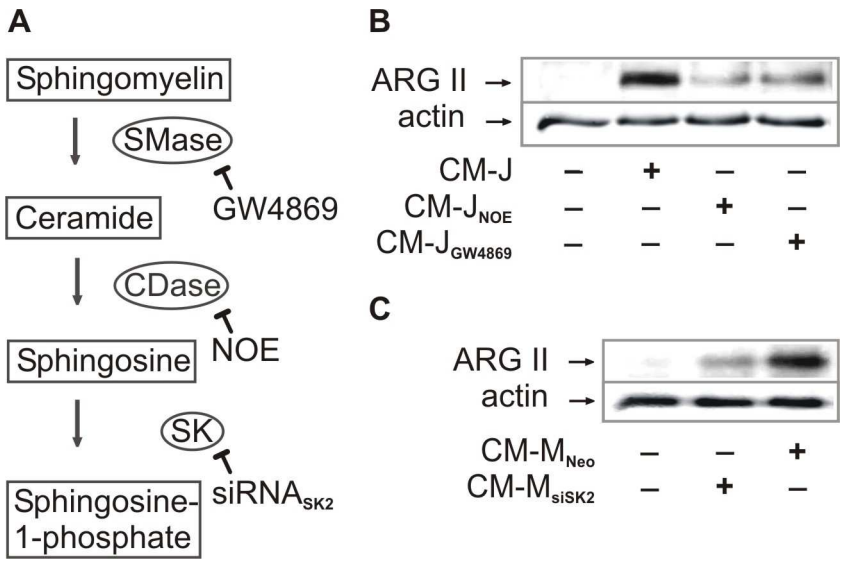

FIGURE 5.3: Contribution of sphingolipids to ARG II induction in MΦs.
(A) Scheme of the sphingolipid metabolism with inhibitors used in this study. (B) Cells were incubated for 16 hours with CM-J or modified conditioned media from Jurkat cells with SMase being inhibited with GW4869 or CDase with NOE. (C) MΦs were stimulated for 16 hours with CM-M_{Neo} from MCF-7 cells with a pSilencer 4.1-CMV neo control vector or CM-M_{siSK2} from MCF-7 cells with a knock-down of SK2. ARG II expression in RAW264.7 cells was determined by Western analysis.

Since the experimental data strongly suggested S1P to be involved in the observed effect, I then analysed the impact of authentic S1P on ARG II expression in MΦs. 0.5 µM authentic S1P had a significant, yet minor effect on ARG II protein expression (FIGURE 5.4 A). Expression increased 2.3 fold compared to controls, while CM-M provoked a 13 fold up-regulation as seen in FIGURE 5.1 A. Furthermore, stimulation of RAW264.7 cells with CM-M_{siSK2} only induced 50% of ARG II protein compared to CM-M_{Neo}. Application of CM-M_{siSK2} in combination with 150 nM S1P restored ARG II expression to levels similar to CM-M_{Neo} (FIGURE 5.4 B).

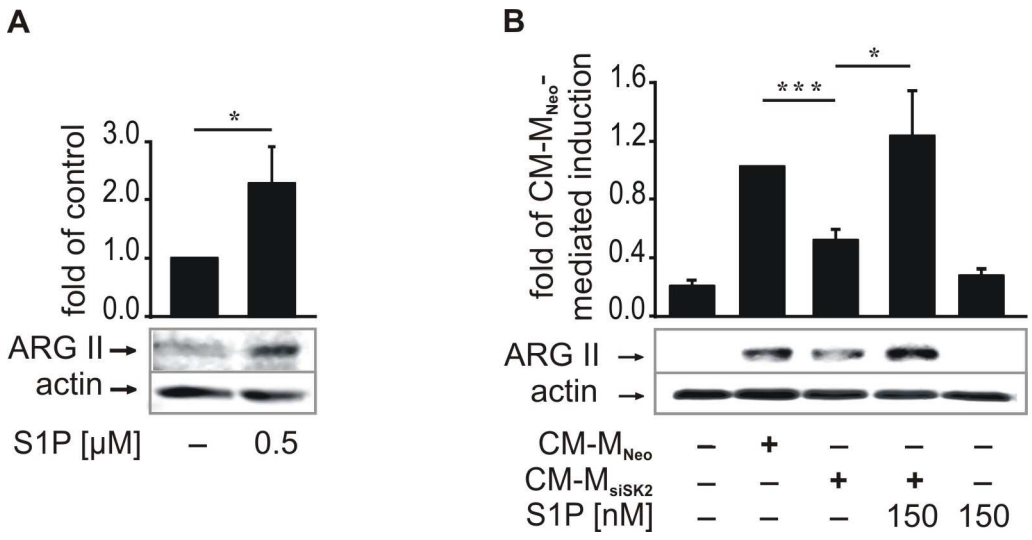

FIGURE 5.4: S1P contributes to ARG II up-regulation.
(A) MΦs were incubated with 0.5 µM authentic S1P. (B) Cells were treated with CM-M_{Neo}, CM-M_{siSK2} with or without 150 nM S1P or with 150 nM authentic S1P alone for 16 hours. ARG II expression in RAW264.7 cells was determined by Western analysis. Graphs show quantifications of ARG II expression. Asterisks mark statistically significant differences. (*) $p \leq 0.05$, (***) $p \leq 0.0005$.

Since authentic S1P had a minor effect on ARG II protein expression compared to CM, I presumed the requirement of at least another, yet unidentified factor for robust expression of ARG II. Furthermore, I either used CM-J at a ratio of 5 ACs to 1 MΦ (5ACs:1MΦ) or a lower ratio of 3 ACs to 1 MΦ, which was less effective at inducing ARG II protein. Addition of different concentrations of authentic S1P to the weaker CM-J stimulus (3ACs:1MΦ) did not restore ARG II expression brought about by the stronger CM-J stimulus (5ACs:1MΦ) (FIGURE 5.5). Thus, the requirement of the postulated second factor was underscored by this observation, since its dilution could not be rescued by S1P.
I concluded that glycerophospholipids, such as LPC and LPA, were not involved, while the sphingolipid S1P was indispensable for ARG II induction.

5.1.3 S1P mediates ARG II up-regulation via $S1P_2$

My data showed that S1P was essential for CM-dependent ARG II induction in MΦs. S1P is a pleiotropic lipid factor with many actions mediated by five receptors coupled to three different types of G proteins with several effector proteins (see 3.4.3.2). Considering the numerous potential mechanisms, the next step consisted in the identification of the receptor on MΦs mediating ARG II up-regulation in order to constrict the possibilities.

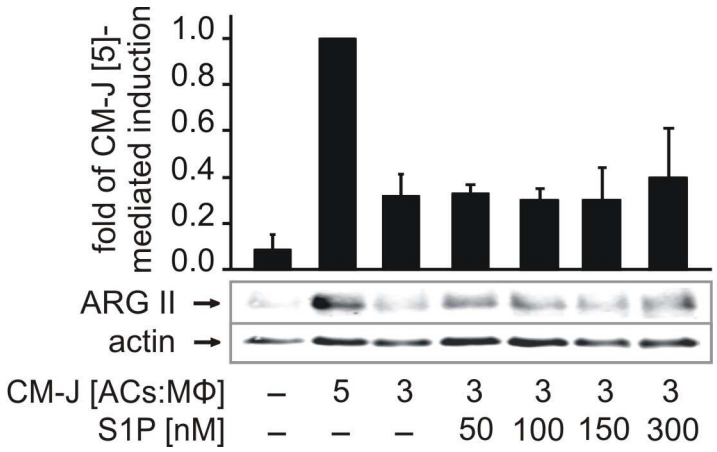

FIGURE 5.5: Sub-optimal concentrations of CM-J cannot be rescued by S1P to restore ARG II up-regulation.
RAW264.7 cells were incubated for 16 hours with CM-J at a ratio of 5 ACs to 1 MΦ (5ACs:1MΦ) or 3 ACs to 1 MΦ (3ACs:1MΦ). CM-J (3ACs:1MΦ) was combined with the indicated concentrations of S1P. ARG II expression was determined by Western analysis. The graph shows quantification of ARG II expression.

It is well established that S1P signals by binding to S1P receptors ($S1P_{1-5}$). Since $S1P_1$ and $S1P_2$ predominate in murine MΦs (Hughes et al., 2008), I employed specific inhibitors at effective concentrations to clarify which receptor was involved in ARG II up-regulation (Balthasar et al., 2008). VPC23019, a $S1P_{1,3}$ antagonist, had no effect (FIGURE 5.6 A), while the $S1P_2$ antagonist JTE013 strongly reduced CM-M-mediated ARG II expression (FIGURE 5.6 B). This result was underscored by siRNA knock-down of $S1P_2$ in RAW264.7 cells (FIGURE 5.6 C). A significant $S1P_2$ knock-down to 60% (black bars) significantly reduced ARG II induction to 70% (white bars) compared to controls. Thus, S1P in CM mediates ARG II up-regulation by triggering $S1P_2$ signaling in MΦs.

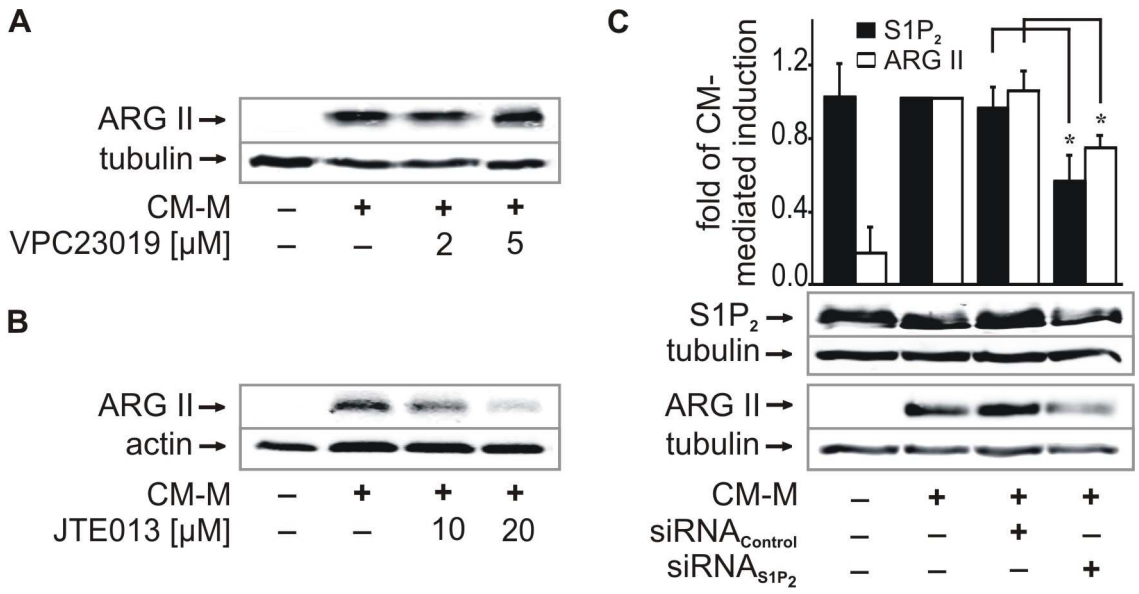

FIGURE 5.6: S1P mediates ARG II up-regulation via $S1P_2$.
(A,B) Cells were incubated for 16 hours with CM-M with or without the specific $S1P_{1,3}$ antagonist VPC23019 or the $S1P_2$ antagonist JTE013 at the indicated concentration. Antagonists were pre-incubated for 45 minutes. (C) MΦs were transfected with siRNA against $S1P_2$ 3 days prior to stimulation with CM-M for 16 hours. ARG II expression and $S1P_2$ knock-down in RAW264.7 cells were determined by Western analysis. The graph shows quantification of the $S1P_2$ knock-down and ARG II expression. Asterisks mark statistically significant differences ($p \leq 0.05$).

5.2 Extracellular signal-regulated kinase 5 (ERK5) contributes to CM-mediated ARG II up-regulation in MΦs

My previous experimental data demonstrated the contribution of $S1P_2$ signaling to ARG II expression in MΦs, thus directing my interest towards potential target effectors.

5.2.1 ERKs contribute to ARG II up-regulation

To determine the signaling pathway activated by $S1P_2$ in more detail, I used specific inhibitors of protein kinases that are well-known as downstream effectors of S1P (FIGURE 5.7) (Kluk and Hla, 2002; Mathieson and Nixon, 2006; Weis et al., 2009). Inhibition of Jak, p38 and PI3K, by the Jak inhibitor 1, SB203580 or LY294002 respectively, did not alter ARG II expression, while U0126, a MEK1/2/5 inhibitor, strongly reduced the CM-J-induced ARG II protein (FIGURE 5.7).

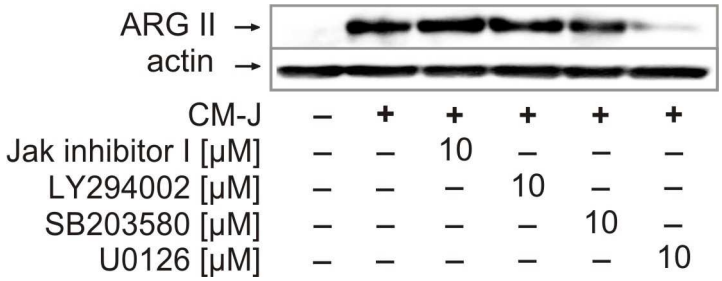

FIGURE 5.7: ERKs contribute to ARG II up-regulation.
RAW264.7 cells were incubated for 16 hours with CM-J and CM-J in combination with the Jak inhibitor I, the PI3K inhibitor LY294002, the p38 inhibitor SB203580 or the MEK1/2/5 inhibitor U0126 at the indicated concentration. Inhibitors were pre-incubated for 45 minutes. ARG II expression in RAW264.7 cells was determined by Western analysis.

5.2.2 ERK5 signaling induces ARG II expression

MEK1/2 are ERK1/2 up-stream kinases, while MEK5 directly activates ERK5. To discriminate between ERK1/2 and ERK5 signaling involved in ARG II up-regulation, I treated RAW264.7 cells with CM-J from apoptotic Jurkat cells where SMase or CDase were inhibited with GW4869 or NOE. Although these modified conditioned media failed to induce ARG II protein, they still induced ERK1/2 phosphorylation (FIGURE 5.8 A). Furthermore, high concentrations of the ERK1/2 inhibitor PD98059 (50 µM) attenuated CM-M-dependent ARG II up-regulation, while lower doses (10 µM) had no effect, despite inhibiting ERK1/2 phosphorylation (FIGURE 5.8 B). These findings ruled out the involvement of ERK1/2 and suggested ERK5 to up-regulate ARG II.

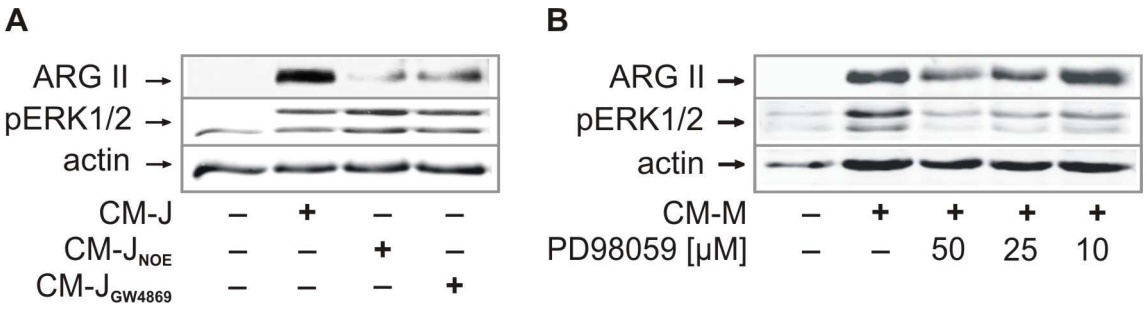

FIGURE 5.8: ERK1/2 is not involved in ARG II up-regulation.
(A) RAW264.7 cells were incubated for 16 hours with CM-J or modified conditioned media from Jurkat cells where SMase or CDase were inhibited with GW4869 or NOE. (B) Cells were incubated for 16 hours with CM-M with or without the ERK1/2 inhibitor PD98059 at the indicated concentrations. The inhibitor was pre-incubated for 45 minutes. ARG II expression and ERK1/2 phosphorylation in RAW264.7 cells were determined by Western analysis.

In order to corroborate the importance of ERK5 in ARG II expression, I performed a siRNA knock-down of ERK5, which was verified by Western analysis. As expected, reducing ERK5 by roughly 50% (black bars) significantly diminished CM-M-mediated ARG II protein expression likewise to approximately 50% (white bars) (FIGURE 5.9). Thus, siRNA knock-down of ERK5 clearly demonstrated the participation of ERK5 signaling in CM-mediated ARG II up-regulation in MΦs.

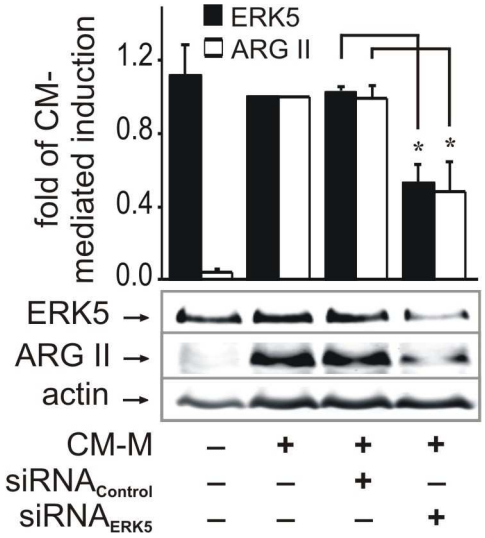

FIGURE 5.9: ERK5 signaling induces ARG II.
Transiently transfected RAW264.7 cells (with siRNA against ERK5) were stimulated with CM-M for 16 hours. ARG II expression and ERK5 knock-down in RAW264.7 cells were determined by Western analysis. The graph shows quantification of the ERK5 knock-down and ARG II expression. Asterisks mark statistically significant differences ($p \leq 0.05$).

5.3 Cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) contributes to ARG II up-regulation in MΦs

Since the induction of ARG II by CM-M was detectable after 4 hours at protein level and increased constantly up to 20 hours (FIGURE 5.1), I assumed transcriptional regulation to be involved. To verify this presumption and to identify a possible transcription factor, I performed an analysis of the murine ARG II promoter (mpARG II) based on luciferase reporter assays, an EMSA analysis and a decoy-approach.

5.3.1 Murine ARG II promoter (mpARG II) analysis

I generated mpARG II constructs by serial deletion of fragments using different restriction enzymes (FIGURE 4.1). RAW264.7 MΦs were transfected with the respective mpARG II constructs (mpARG II-1856 bp, -1359 bp, -442 bp or -262 bp), further cultured for 24 hours and stimulated with CM-J or 0.5 µM S1P for 16 hours prior to reporter analysis. In agreement with my assumption that transcriptional regulation was taking place, CM-J induced mpARG II activity about 8 fold and S1P about 1.5 fold compared to unstimulated controls. Interestingly, all promoter constructs including the smallest one of 262 bp still displayed CM-J-mediated ARG II promoter activity comparable to the whole promoter (FIGURE 5.10), implying that the responsible transcription factor binds to the 262 bp fragment of mpARG II and thereby enhances ARG II transcription.

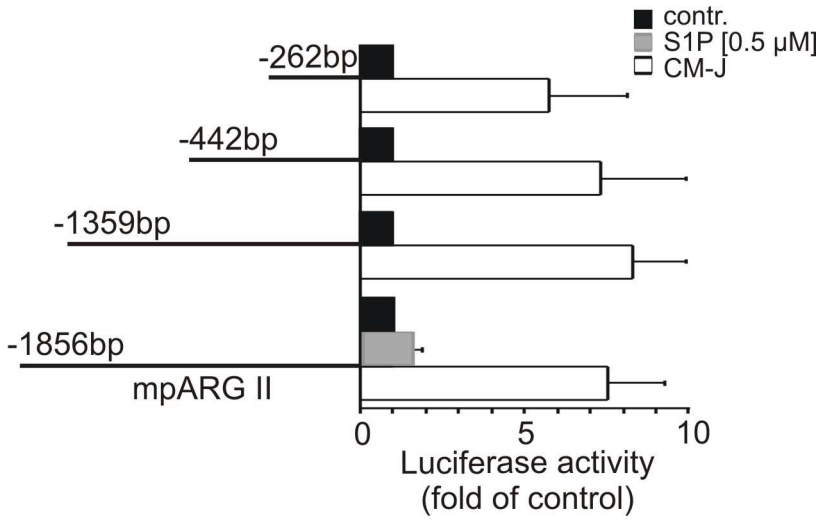

FIGURE 5.10: mpARG II analysis.
Transfected RAW264.7 cells were stimulated for 16 hours with CM-J or 0.5 µM S1P. mpARG II activity was measured by luciferase reporter assay.

5.3.2 CREB contributes to CM-mediated ARG II up-regulation

The next step consisted in the identification of the transcription factor mediating CM-dependent ARG II transcriptional up-regulation in MΦs. For this purpose, I performed *in silico* analysis of the 262 bp fragment of the mpARG II, which revealed, among others, a CREB binding site (FIGURE 5.11).

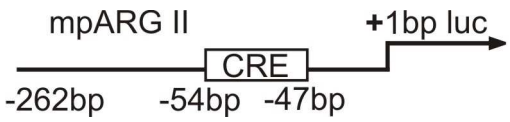

FIGURE 5.11: 262 bp fragment of mpARG II containing a CREB binding site.

Considering that this transcription factor can in fact be activated by ERK5 (Sharma and Goalstone, 2005; Watson et al., 2001), I decided to analyze the effect of CM on CREB binding to the sequence of mpARG II. Therefore, I incubated RAW264.7 cells with CM-M for 16 hours, isolated nuclear proteins followed by EMSA analysis. Employing oligonucleotides containing the mpARG II CRE site showed significantly enhanced binding of CREB in response to CM-M (1.8 fold compared to controls) in EMSA. This result was reinforced by supershift analysis using an anti-CREB-1 Ab (FIGURE 5.12 A). In accompanying competition assays, an excess of unlabeled oligonucleotides (25, 50 or 100 fold excess of unlabeled vs. labeled oligonucleotides) completely abolished the binding signal, while unlabeled oligonucleotides containing a mutation in the CRE site had no effect (FIGURE 5.12 B).

In order to definitely demonstrate the involvement of CREB in ARG II induction, I used a decoy-approach to scavenge active CREB in RAW264.7 MΦs prior to its binding to the promoter of its target genes. I transfected RAW264.7 cells with 3 µM oligonucleotides with the sequence of mpARG II, containing a mutated vs. a non-mutated CREB binding site. After culturing for another 48 hours, RAW264.7 cells were stimulated with CM-M for 16 hours and ARG II expression was subsequently analyzed by Western blotting. In line with my previous observations, decoy-oligonucleotides, which scavenged active CREB, blocked CM-M-mediated ARG II protein induction. The mutated decoy-oligonucleotides did not alter ARG II expression (FIGURE 5.13).

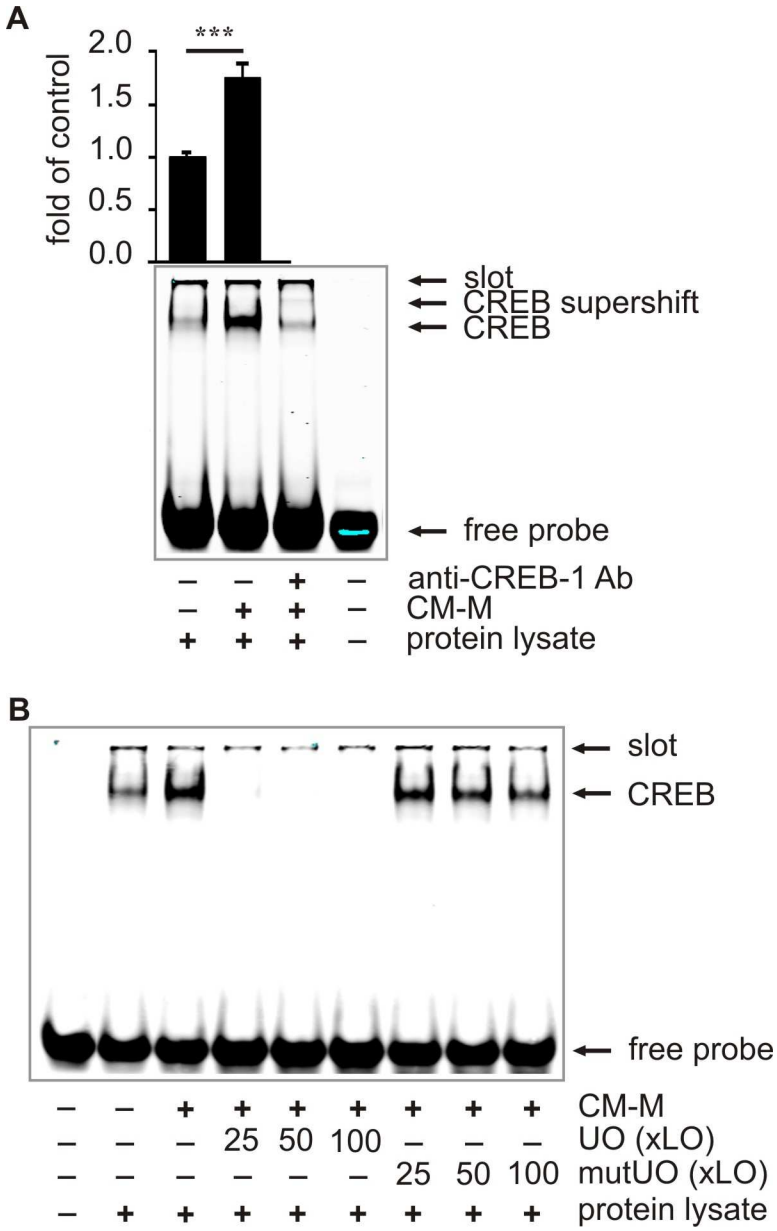

FIGURE 5.12: Enhanced CREB binding to mpARG II oligonucleotides.
(A,B) RAW264.7 cells were stimulated with CM-M for 16 hours. CM-M-mediated CREB binding to mpARG II-oligonucleotides was analysed by EMSA. In (A), a supershift was performed with an anti-CREB-1 Ab. The graph shows quantification of CREB binding to respective oligonucleotides. Asterisks mark statistically significant differences ($p \leq 0.0005$). In (B), competition assays were performed with non-mutated (UO) or mutated unlabeled oligonucleotides (mutUO) in 25, 50 or 100 fold excess over labeled ones (xLO).

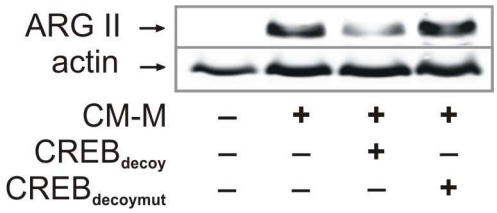

FIGURE 5.13: CREB decoy in RAW264.7 MΦs prevents ARG II induction.
Cells were transiently transfected with mutated or non-mutated CREB decoy-oligonucleotides 48 hours prior to stimulation with CM-M for 16 hours. ARG II expression was detected by Western analysis.

Considering the data obtained by mpARG II and EMSA analysis as well as the CREB-decoy approach, I conclude that CM enhances CREB activity in MΦs thereby inducing the transcription of ARG II.

5.4 $S1P_2$ and ERK5 are required for CREB activation and subsequent ARG II up-regulation in MΦs

My experimental data so far showed that CM-triggered $S1P_2$ and ERK5 signaling as well as CREB DNA-binding and transactivation induced ARG II expression, but a connection between these single components of the putative signaling cascade was still missing. To investigate a possible link between $S1P_2$ signaling and ERK5 phosphorylation in the cascade provoking ARG II up-regulation, I analyzed the effect of the $S1P_2$ antagonist JTE013 on CM-mediated ERK5 activation/phosphorylation. Furthermore, I also investigated the effect of the $S1P_2$ antagonist JTE013 and the MEK5 inhibitor U0126 on CM-induced CREB DNA-binding activity to complete the intermolecular relations.

5.4.1 $S1P_2$ mediates ERK5 phosphorylation

First, I intended to investigate a possible connection between $S1P_2$ and ERK5 by analyzing the impact of CM-triggered $S1P_2$ signaling on ERK5 phosphorylation. However, Western analysis of endogenous ERK5 phosphorylation is known to be difficult. Indeed, I observed a CM-M-dependent induction of ERK5 phosphorylation in RAW264.7 MΦs but detection signals were very weak (FIGURE 5.14).

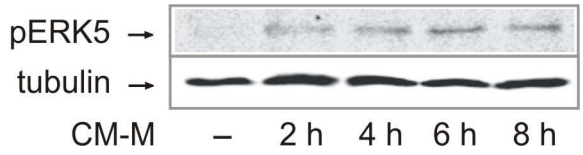

FIGURE 5.14: CM-M induces ERK5 phosphorylation in RAW264.7 MΦs.
Cells were incubated with CM-M for indicated time periods. ERK5 phosphorylation in RAW264.7 cells was determined by Western analysis.

In order to bypass this experimental inconvenience, I over-expressed MEK5, the up-stream kinase of ERK5 in the wild type or constitutively active form, in combination with an ERK5 form containing the activation motif targeted by active MEK5. I performed this over-expression experiment in HEK 293 cells, whose transfection can be achieved with high efficiency compared to MΦs. Given that I wanted to analyze the link between $S1P_2$ signaling and ERK5, HEK 293 cells are appropriate for this approach because, like MΦs, they express $S1P_{1-3}$ (Meyer zu Heringdorf et al., 2001). I transiently transfected these cells with different expression plasmids encoding HA-tagged ERK5N (truncated form of ERK5 (residues 1-407) containing the TEY activation motif targeted by MEK5), FLAG-tagged MEK5 WT (wild type form of the up-stream kinase MEK5) or MEKD (constitutively active form of MEK5). Over-expression of HA-ERK5N, FLAG-MEK5 WT and FLAG-MEK5D was confirmed by Western analysis. Co-expression of HA-ERK5N and constitutive active FLAG-MEK5D was used as a positive control and resulted in the strongest phosphorylation signal of ERK5N (FIGURE 5.15 lane 9). HEK 293 cells co-expressing HA-ERK5N and FLAG-MEK5 WT were stimulated with CM-M. CM-M time-dependently induced ERK5N phosphorylation, starting at 1 hour and increasing up to 8 hours (FIGURE 5.15 lanes 3-7). ERK5N phosphorylation was normalized to the sum of the expression of ERK5N and MEK WT [pERK5/(MEK5+ERK5)]. Enhanced ERK5N phosphorylation was initiated by $S1P_2$ since the specific antagonist JTE013 [10 µM] significantly reduced CM-M-mediated ERK5N phosphorylation (FIGURE 5.15 lane 8). Furthermore, 0.5 µM authentic S1P also induced ERK5N phosphorylation, which peaked after 6 hours of stimulation (FIGURE 5.16).

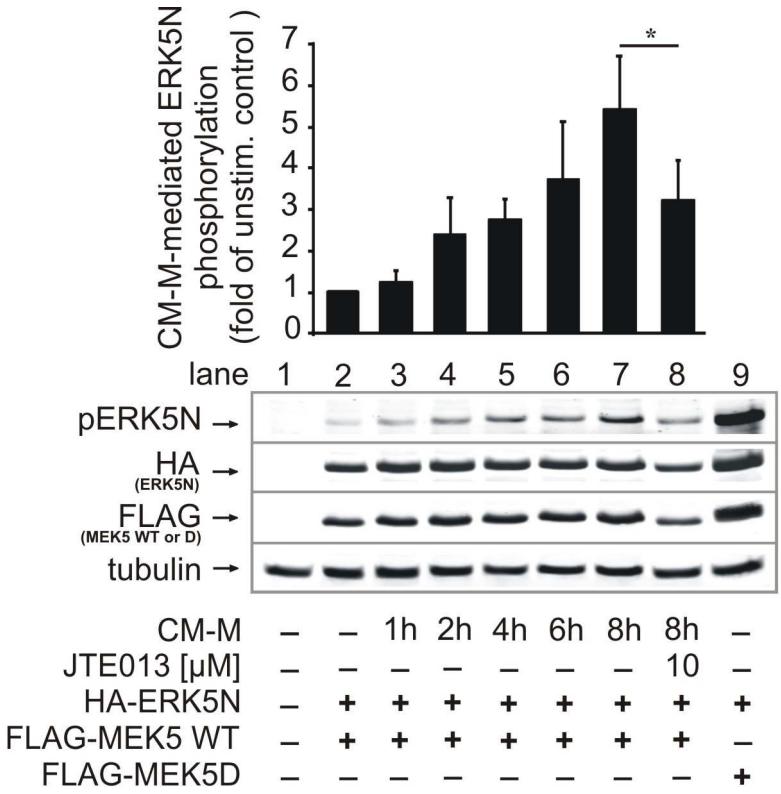

FIGURE 5.15: $S1P_2$ mediates ERK5N phosphorylation.
HEK 293 cells were transiently co-transfected with pcDNA3-HA1-ERK5N and pcDNA3-FLAG1-MEK5 WT or pcDNA3-FLAG1-MEK5D and kept in FCS-free medium overnight. Cells were then incubated with CM-M for the indicated time with or without the $S1P_2$ antagonist JTE013. ERK5N phosphorylation, HA-tag and FLAG-tag over-expression were analyzed by Western blotting. The graph shows quantification of CM-M-mediated ERK5N phosphorylation normalized to the sum of the expression of ERK5N and MEK WT [pERK5/(MEK5+ERK5)]. Asterisk marks statistically significant differences ($p \leq 0.05$).

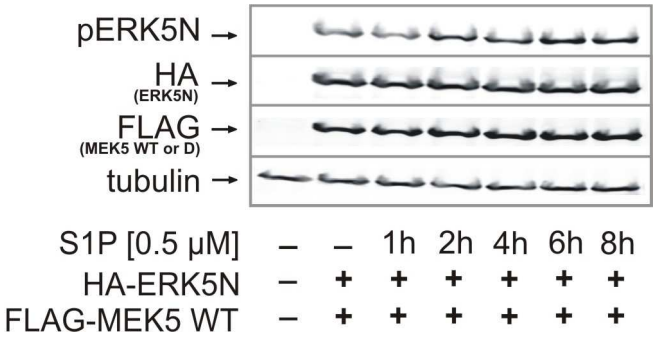

FIGURE 5.16: Authentic S1P provokes ERK5N phosphorylation.
HEK 293 cells were transiently co-transfected with pcDNA3-HA1-ERK5N and pcDNA3-FLAG1-MEK5 WT and kept in FCS-free medium overnight. Cells were then incubated with 0.5 µM S1P for the indicated time. ERK5N phosphorylation, HA-tag and FLAG-tag over-expression were analyzed by Western blotting.

5.4.2 $S1P_2$ and ERK5 induce CREB binding to the target sequence in mpARG II

The link between $S1P_2$, ERK5 and CREB activation had to be confirmed by EMSA analysis. The $S1P_2$ antagonist JTE013 and U0126, the inhibitor of the up-stream kinase of ERK5, reduced CREB binding to oligonucleotides containing the mpARG II CRE site after 8 as well as 16 hours (FIGURE 5.17 A,B) demonstrating the importance of $S1P_2$ and ERK5 signaling for CREB activation.

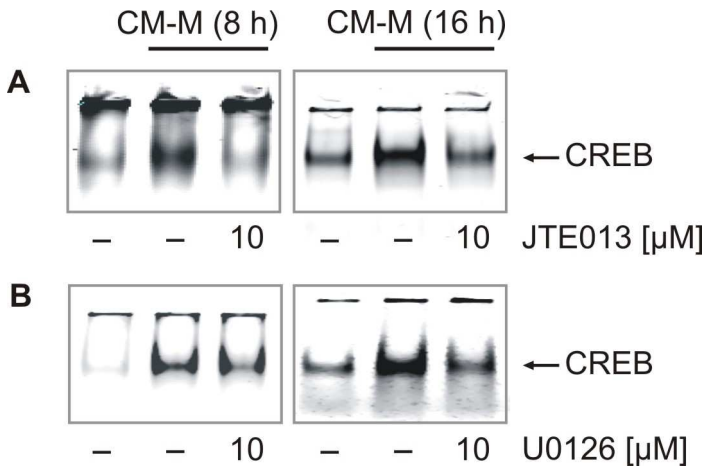

FIGURE 5.17: $S1P_2$ and MEK5 provoke CREB binding to target sequence.
(A,B) RAW264.7 MΦs were incubated with CM-M with or without the $S1P_2$ antagonist JTE013 or the MEK inhibitor U0126 at the indicated concentration for 8 or 16 hours. JTE013 and U0126 were pre-incubated for 45 minutes. CREB DNA-binding was determined by EMSA.

Furthermore, a physiological relevance of $S1P_2$ and ERK5 signaling was analyzed by using primary peritoneal MΦs from C57BL/6 mice. Similar to the effect in RAW264.7 MΦs, CM-M also up-regulated ARG II protein expression in primary murine MΦs. Furthermore, CM-M-mediated ARG II up-regulation in peritoneal murine MΦs was reduced as expected by JTE013 as well as U0126 (FIGURE 5.18). However, in contrast to RAW264.7 MΦs these primary cells already express basal levels of ARG II protein.

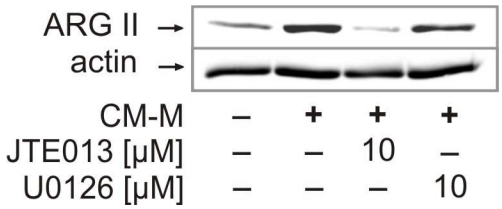

FIGURE 5.18: Physiological relevance of $S1P_2$ and ERK5 signaling.
Peritoneal MΦs from C57BL/6 mice were incubated with CM-M with or without the $S1P_2$ antagonist JTE013 or the MEK inhibitor U0126 at the indicated concentration for 16 hours. JTE013 and U0126 were pre-incubated for 45 minutes. ARG II expression was detected by Western analysis.

Considering my experimental data, I conclude that S1P in CM contributes to ARG II up-regulation in MΦs by binding to $S1P_2$, subsequently inducing ERK5 signaling and provoking CREB activation.

5.5 Mechanism of ERK5-dependent CREB activation in MΦs

Finally, I was interested in the activation mechanism of CREB by ERK5. ERKs are proline-directed kinases that cannot directly phosphorylate CREB at serine 133. Instead, they induce CREB phosphorylation by activating an intermediate kinase of the ribosomal S6 kinase (RSK) family (Watson et al., 2001) or by influencing the activation of PKA through the regulation of PDE4D3 and cAMP hydrolysis (Dodge-Kafka et al., 2005). In order to evaluate these two options, I decided first to analyze the importance of cAMP signaling for CM-M-mediated ARG II up-regulation in MΦs, since recently alveolar MΦs were shown to display elevated levels of cAMP in response to ACs (Medeiros et al., 2009) and cAMP/CREB signaling was reported to account for the alternative regulatory phenotype of MΦs (Bystrom et al., 2008; Ruffell et al., 2009).

5.5.1 cAMP signaling contributes to CM-mediated ARG II induction

To investigate the role of cAMP in ARG II up-regulation in MΦs, I inhibited the cAMP-producing enzyme adenylyl cyclase. RAW264.7 cells were stimulated with CM-M for 16 hours and adenylyl cyclase was inhibited by the specific inhibitor SQ22536, which had been pre-incubated for 45 minutes. ARG II was then analyzed by Western blot. Inhibition of adenylyl cyclase and subsequent cAMP production impaired CM-M-mediated ARG II up-regulation (FIGURE 5.19).

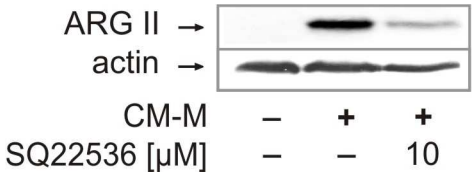

FIGURE 5.19: Adenylyl cyclase contributes to ARG II up-regulation.
RAW264.7 cells were incubated for 16 hours with CM-M or CM-M in combination with the adanylyl cyclase inhibitor SQ22536 at the indicated concentration. SQ22536 was pre-incubated for 45 minutes. ARG II expression in RAW264.7 cells was determined by Western analysis.

Thus, cAMP production in response to CM seems important for ARG II induction in MΦs. Taking this into consideration, I went on with the analysis of PKA participation since this kinase is the main target of cAMP. In accordance to the previous results, inhibition of the cAMP activated PKA by the specific inhibitor KT5720 also prevented CM-M-mediated ARG II up-regulation, convincingly pointing to the contribution of cAMP and PKA signaling (FIGURE 5.20).

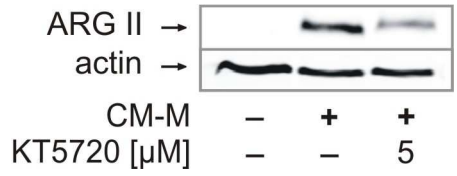

FIGURE 5.20: PKA contributes to ARG II up-regulation.
RAW264.7 cells were incubated for 16 hours with CM-M or CM-M in combination with the PKA inhibitor KT5720 at the indicated concentration. KT5720 was pre-incubated for 45 minutes. ARG II expression in RAW264.7 cells was determined by Western analysis.

5.5.2 Combined cAMP elevation and phosphodiesterase 4 (PDE4) inhibition mimic the CM effect on ARG II expression

CM-dependent ARG II up-regulation in MΦs requires cAMP/PKA as well as ERK5 signaling, so the next step consisted in elucidating the link between these two pathways. Considering that ERK5 has been shown to inhibit the cAMP-hydrolizing enzyme PDE4D3, and consequently to enhance PKA activity by augmenting cAMP availability (Dodge-Kafka et al., 2005), I presumed that activation of cAMP production with the adenylyl cyclase activator forskolin and at the same time inhibition of PDE4 with rolipram would mimic CM-mediated

ARG II up-regulation. In fact, enhanced cAMP production by forskolin in RAW264.7 MΦs was sufficient to induce ARG II protein, while inhibition of PDE4 with rolipram alone had no effect (FIGURE 5.21). This experiment shows that basal cAMP levels are not sufficient for ARG II up-regulation, since inhibition of PDE4 did not induce ARG II, and suggests that a cAMP elevating stimulus is necessary. Furthermore, forskolin-mediated ARG II up-regulation was strongly enhanced by rolipram, suggesting that cAMP/PKA signaling and PDE4 inhibition by ERK5 may mediate ARG II up-regulation in my experimental system. Although forskolin and rolipram provoke a similar effect, CM-M still shows a stronger induction of ARG II protein expression.

FIGURE 5.21: Elevation of cAMP combined with PDE4 inhibition mimic the CM-M effect on ARG II expression.
RAW264.7 cells were incubated for 16 hours with the adenylyl cyclase activator forskolin, the PDE4 inhibitor rolipram or both at the indicated concentration, and with CM-M. ARG II expression in RAW264.7 cells was determined by Western analysis.

Along this line, rolipram enhanced CM-M-mediated ARG II up-regulation (FIGURE 5.22) further indicating that regulation of PDE4 contributes to ARG II induction.

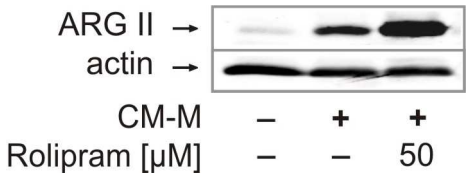

FIGURE 5.22: Rolipram enhances CM-mediated ARG II induction.
RAW264.7 cells were incubated for 16 hours with CM-M or CM-M in combination with the PDE4 inhibitor rolipram at the indicated concentration. Rolipram was pre-incubated for 45 minutes. ARG II expression in RAW264.7 cells was determined by Western analysis.

5.5.3 CM induces cAMP accumulation in RAW264.7 MΦs

Finally, since cAMP elevation seemed a prerequisite for ARG II up-regulation I investigated the impact of CM-M on cAMP levels in RAW264.7 MΦs. As expected, CM-M provoked cAMP accumulation in these cells (FIGURE 5.23 A). The CM-M-dependent transient raises of cAMP levels in the first 2 hours of stimulation clearly match the assumption that elevation of cAMP activates PDE4 in a negative feedback loop leading to its hydrolysis. At later time points, corresponding to CM-M-mediated ERK5 phosphorylation/activation patterns, PDE4 seems to be inhibited thereby allowing a consistent increase in cAMP levels. Furthermore, maximal CM-M-dependent cAMP accumulation (7.4 pmol/ml) lay in the same range as maximal forskolin/IBMX-mediated cAMP elevation (7.7 pmol/ml) (FIGURE 5.23 B).

I hypothesize that a yet unidentified factor in CM mediates cAMP elevation, which induces PKA activation and consequently CREB phosphorylation in MΦs. At the same time, S1P-mediated ERK5 activation provokes PDE4 inhibition, thereby sustaining cAMP signaling.

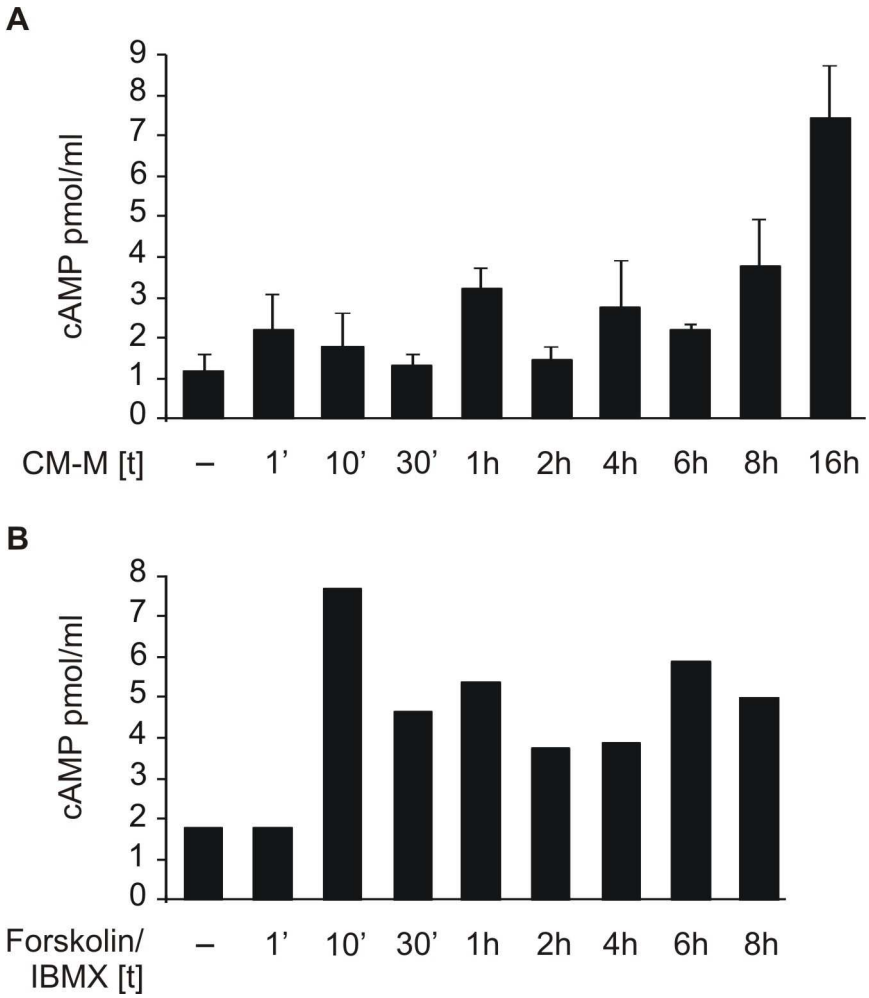

FIGURE 5.23: CM-M provokes a consistent increase of cAMP in RAW264.7 cells. (A) MΦs were incubated for the indicated times with CM-M (2 independent experiments, in each case duplicates). (B) Cells were incubated for the indicated times with the adenylyl cyclase activator forskolin (20 µM) and the nonselective PDE inhibitor IBMX (0.5 mM). IBMX was pre-incubated for 45 minutes (1 experiment, duplicates). cAMP levels were determined by EIA.

6 Discussion

The remarkable plasticity of macrophages (MΦs) allows them to respond adequately to many different environmental stimuli. Accordingly, MΦs evolve into populations with heterogeneous phenotypes and complex functions in tissue homeostasis as well as in innate and acquired immunity. During host defence, MΦs are classically activated and develop e.g. cytotoxic properties, among which enhanced NO production is prominent (Mosser and Edwards, 2008). However, excessive formation of the cytotoxic NO is detrimental for the surrounding tissue. In order to dampen and resolve inflammation, MΦs adopt an alternative activated phenotype characterized by impaired NO formation. In this context, arginase (ARG) expression has been shown to modulate NO production by competing with iNOS for the common substrate L-arginine (Freire-de-Lima et al., 2006; Gotoh and Mori, 1999; Johann et al., 2007; Morris, 2004; Topal et al., 2006). Although, there are different opinions concerning the mechanism mediating ARG-dependent impaired NO formation (see 3.4.2), ARG II seems to account for an early and ARG I probably for a late modulation of NO production and a possible explanation will be discussed later (see 6.1.4).

Recently, Johann *et al.* demonstrated that pre-incubation of RAW264.7 MΦs with apoptotic cell (AC)-conditioned medium (CM) impaired IFNγ-mediated NO generation by up-regulating ARG II (Johann et al., 2007). MΦs incubated with ACs have been reported to undergo a regulatory-like activation (Fadok et al., 1998). More precisely, I assume that ACs activate MΦs alternatively thereby engendering a regulatory-like phenotype that shares characteristics with wound-healing MΦs. Indeed, IL-4 and IL-13 induce wound-healing and IL-10 the development of regulatory MΦs by definition (see FIGURE 3.3). Since ACs secrete IL-10 (Gao et al., 1998) as well as IL-4 (Hodge et al., 2002), MΦs exposed to them are likely to adopt a phenotype between wound-healing and regulatory MΦs (FIGURE 6.1). These regulatory-like MΦs may participate in resolution of inflammation as well as in tissue remodeling.

Since ARG expression is a hallmark of the regulatory MΦ phenotype (Mantovani et al., 2004b), I assumed that elucidation of the signaling pathway(s) mediating ARG II up-regulation in MΦs could provide general information on

intracellular mechanisms responsible for the development and maintenance of the regulatory MΦ phenotype.

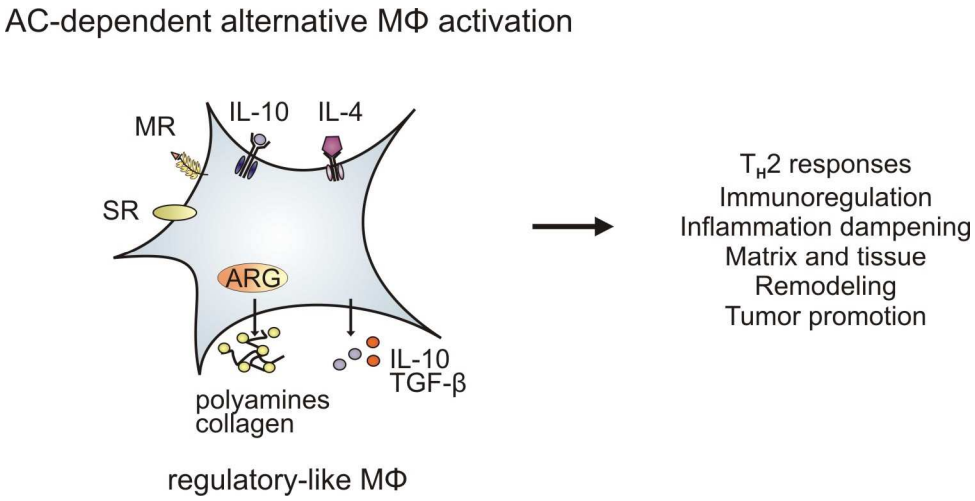

FIGURE 6.1: Postulated regulatory-like MΦ phenotype induced by ACs.
Alternative M2 MΦ activation is heterogenous and raises different M2 MΦ phenotypes. Interleukin 4 (IL-4) and IL-13 induce wound-healing while IL-10 generates regulatory MΦs. Since ACs secrete IL-10 as well as IL-4, MΦs exposed to them are likely to adopt a phenotype between wound-healing and regulatory MΦs characterized by exibition of scavenger (SR) and mannose receptors (MR), release of immunoregulatory factors, like transforming growth factor β (TGF-β) and IL-10, up-regulation of arginase (ARG) and consequent production of polyamines and the matrix component collagen.

Worth mentioning in this context is the fact that TAMs have a phenotype similar to MΦs treated with ACs (Mantovani et al., 2004a). Thus, ARG II might contribute to their pro-tumorigenic properties by impairing NO production. In addition, I speculate that the ARG II product L-ornithine, as a precursor of polyamines which are secreted by TAMs (Mantovani et al., 2004a), could also facilitate tumor growth. In fact, ARG II has also been reported to be highly expressed in some tumors, especially in kidney, lung, breast and prostate cancer cells (Mumenthaler et al., 2008; Porembska et al., 2003; Rotondo et al., 2008; Tate et al., 2008) and to metabolize L-arginine thereby supplying tumors with L-ornithine needed for their rapid growth and at the same time inducing T cell dysfunction (Singh et al., 2000; Tate et al., 2008). This T cell dysfunction arises from L-arginine depletion and subsequent impaired CD3ζ expression, which is a key element for T cell function. Thus, ARG II might favor tumor development in three different ways, first by preventing tumoricidal MΦ activity,

second by supplying the tumor with polyamines (derived from tumor cells or MΦs) and third by promoting immune escape.

Considering the important contribution of ARG II in the regulatory MΦ phenotype and in cancer development, as well as the lack of information on the molecular mechanisms of its expression, I aimed at elucidating the signaling pathway to understand how CM induces ARG II.

6.1 Mechanism mediating ARG II up-regulation in MΦs in response to CM

6.1.1 AC-derived S1P contributes to ARG II up-regulation

Digestion and denaturation of proteins in CM with proteinase K and heat inactivation (100℃) did not impair ARG II protein expression in MΦs, indicating that a lipid rather than a protein up-regulated ARG II. Various bioactive glycerophospholipids and sphingolipids, like IP_3, LPC, LPA, ceramide, ceramide 1-phosphate (C1P), sphingosine or S1P are involved in cellular signaling (Hannun and Obeid, 2008; Xu et al., 2003), some of which have been reported to be released from ACs (Lauber et al., 2003; Weigert et al., 2006). To characterize the ARG II promoting lipid factor(s) present in CM, I decided to sequentially inhibit lipid metabolizing enzymes that generate bioactive lipids. Glycerophospholipids produced by ACs apparently are not involved because blocking PLA_2 or PLC left ARG II expression unaltered. In contrast, blocking the enzymes of the sphingolipid metabolism SMase and CDase in ACs showed significant effects. These observations pointed to the importance of sphingolipids and in particular S1P in CM-mediated ARG II induction, since ceramide and sphingosine are S1P precursors. The importance of S1P was underscored by using modified CM from MCF-7 cells with a stable SK2 knock-down, which contained a reduced S1P amount in comparison to normal CM (Weigert et al., 2007). Indeed, induction of ARG II by CM_{siSK2} was minor and could be restored by adding 150 nM S1P. Moreover, 0.5 µM authentic S1P induced ARG II promoter activity and protein expression. As this was weak compared to the action of CM, this observation suggested the involvement of at least another factor secreted/released from ACs. This unidentified factor either directly co-operated with S1P to amplify S1P-signaling or indirectly stimulated

autocrine signaling by MΦs. Furthermore, sub-optimal concentrations of CM were less effective at inducing ARG II protein and S1P did not restore ARG II expression brought about by optimal ones. Thus, the requirement of the postulated second factor was underscored by this observation, since its dilution could not be rescued by S1P. I can exclude protein factors since CM lacking functional proteins still induced ARG II, but have to consider lipids, carbohydrates as well as nucleosides. C1P could be one candidate since it has been shown to act synergistically with S1P in human alveolar epithelial cells. S1P and C1P, while having minor effects by themselves, potentiated production of PGE_2 (Pettus et al., 2004). This would fit to the observation I made with CM_{GW4869}, depleted in ceramide (precursor of C1P), whose ability to up-regulate ARG II in MΦs was strongly impaired. Based on simple pharmacologic inhibition of SK with dimethylsphingosine (DMS), Johann *et al.* originally excluded S1P as a mediator of ARG II expression (Johann et al., 2007). However, DMS is rather unspecific and e.g. causes activation of the epidermal growth factor (EGF) receptor (Igarashi et al., 1990), which in turn can activate ERK5 signaling (Scapoli et al., 2004) leading to deceiving assumptions.

6.1.2 S1P contributes to ARG II expression by activating $S1P_2$

S1P signaling is mediated by activation of specific G protein-coupled receptors. Five S1P receptors ($S1P_{1-5}$) are identified up to date. MΦs predominantly express $S1P_1$, $S1P_2$, and to a much lesser extent $S1P_3$ (Goetzl et al., 2004). Furthermore, only $S1P_1$ and $S1P_2$ have been reported in murine MΦs (Hughes et al., 2008).

Antagonizing $S1P_{1,3}$ with VPC23019 did not seem to alter ARG II expression, while JTE013-mediated antagonism as well as siRNA knock-down of $S1P_2$ demonstated that this receptor signals towards ARG II induction. Similar to my observations, Jiang *et al*. reported that S1P signals through $S1P_2$ in RAW264.7 MΦs thereby enhancing cellular responses triggered by isoproterenol and PGE_2, while having a minor effect by itself (Jiang et al., 2007). Thus, synergistic signaling of S1P with other mediators does not seem unusual. Further experiments should consider MΦ-derived PGE_2 and IL-10 as potential unidentified second factors, as these mediators are known to induce ARG II as well as to enhance signaling of S1P or isoproterenol (Barksdale et al., 2004;

Corraliza and Moncada, 2002; Jiang et al., 2007). Based on pharmacological inhibition of COX-2, which failed to restore NO production in MΦs, Johann *et al.* previously considered MΦ-derived PGE_2 unlikely to contribute to CM-mediated ARG II up-regulation (Johann et al., 2007). However, low levels of PGE_2 derived from COX-1 may be sufficient to synergize with S1P.

6.1.3 ERK5 signaling accounts for CM-mediated ARG II induction

Pharmacological inhibition of well-known downstream effector kinases of S1P revealed that inhibition of MEK1/2/5, which are direct up-stream kinases of ERK1/2/5, prevented CM-mediated ARG II induction. A direct role of ERK1/2 was ruled out, since modified conditioned media that failed to induce ARG II still allowed phosphorylation of ERK1/2. Furthermore, low concentrations of the ERK1/2 inhibitor PD98059 prevented CM-mediated ERK1/2 phosphorylation but did not attenuate ARG II expression. Therefore, I assumed that ERK5 signaling was mandatory for CM-mediated ARG II up-regulation. This assumption was corroborated by siRNA knock-down of ERK5, which significantly reduced ARG II expression. In addition to the similarity of their activation motif (Thr-Glu-Tyr), ERK5 and ERK1/2 share some actions in the regulation of cell proliferation and differentiation. However, unlike other MAP kinases, ERK5 does not only transmit signals to downstream molecules by phosphorylation but also contains a potent transcriptional activation domain within its C-terminus (Morimoto et al., 2007).

Previous studies revealed several ERK5-mediated cellular responses in e.g. cancer (Esparis-Ogando et al., 2002; McCracken et al., 2008), survival or cell proliferation (Scapoli et al., 2004; Watson et al., 2001). However, there is little known about the role of ERK5 in MΦs. One study reported LPS-mediated activation of ERK1/2, c-Jun N-terminal kinase/stress-activated protein kinase (JNK), p38 and ERK5, which synergistically induced TNF-α gene expression in MΦs (Zhu et al., 2000). Furthermore, ERK5 is indispensable for optimal colony-stimulating factor 1 (CSF-1)-induced survival and proliferation of monocytes and MΦs (Rovida et al., 2008). Thus, my results suggest an interesting novel role of ERK5 in regulatory MΦ activation, since ARG expression is a hallmark of the regulatory phenotype. A further hint for the importance of ERK5 in alternative MΦ activation was provided by a recent study showing an AC-dependent

induction of PPARδ in MΦs, which functioned as transcriptional sensor of dying cells. The authors reported that MΦs from PPARδ knock-out mice were impaired in AC clearance due to lower opsonin production. Furthermore, ACs failed to suppress the release of pro-inflammatory and to enhance the secretion of anti-inflammatory cytokines in these MΦs (Mukundan et al., 2009). Since ERK5/PPARδ association has been shown to enhance PPARδ transcriptional activity in a myoblast cell line (Woo et al., 2006), one could speculate that ERK5-dependent PPARδ activation is a further mechanism contributing to regulatory MΦ activation. These reports, along with my data, strongly suggest a crucial role of ERK5 for the development and maintenance of the regulatory MΦ phenotype.

In addition, the simultaneous up-regulation of ERK5 and ARG II in breast and prostate cancer might suggest ERK5-mediated induction of ARG II also in these cases (Esparis-Ogando et al., 2002; McCracken et al., 2008; Mumenthaler et al., 2008; Porembska et al., 2003).

6.1.4 CREB DNA-binding and transactivation is necessary for CM-dependent ARG II up-regulation

CM-dependent ARG II expression seemed to be dependent on transcriptional regulation since the protein accumulated from 4 hours up to 20 hours. This assumption was substantiated by luciferase reporter analysis of the murine ARG II promoter (mpARG II). Furthermore, promoter deletion analysis showed a 262 bp fragment up-stream of the start codon to be needed for luciferase reporter expression. *In silico* analysis of the 262 bp fragment revealed binding sites for several transcription factors, including HIF-1, GATA-1 and CREB. There have been several reports showing that CREB is activated by ERK5 signaling (Sharma and Goalstone, 2005; Watson et al., 2001). Watson *et al.* reported that the ERK5 pathway, triggered by the neurotrophin receptor, was necessary for CREB-mediated survival of neurons. The same authors proved that the expression of a dominant-negative MEK5 prevented neurotrophin-mediated ERK5 activation as well as CREB phosphorylation. Based on these observations, I investigated the impact of CREB in CM-mediated ARG II up-regulation. Indeed, EMSA, supershift analysis and even more importantly

scavenging active CREB in RAW264.7 MΦs by decoy-oligonucleotides, demonstrated its importance for CM-mediated ARG II induction.

Furthermore, previous studies reported that ARG II mRNA is up-regulated by 8-bromo-cAMP or dibutyryl-cAMP, thus pointing to the implication of CREB, even though the molecular mechanisms were not elucidated (Corraliza and Moncada, 2002; Morris et al., 1998). Along this line, activation of adenylyl cyclase with 100 µM forskolin, which is known to raise cAMP levels, up-regulated ARG II protein in RAW264.7 MΦs in my experiment. A recent study on tissue regeneration demonstrated the importance of CREB for the anti-inflammatory M2 gene expression in MΦs (Ruffell et al., 2009). CREB induced CCAAT/enhancer binding protein β (C/EBPβ) expression in these cells, which subsequently up-regulated IL-10, IL-13 receptor and ARG I. Thus, this might be the explanation for an early ARG II and a late ARG I-dependent NO modulation, since my data showed a rapid CREB-mediated ARG II induction, while Ruffell *et al.* reported that ARG I up-regulation required antecedent CREB-dependent enhanced C/EBPβ expression.

6.1.5 $S1P_2$ triggers ERK5 signaling and subsequent CREB activation resulting in ARG II induction

In order to link the observed signaling events mediated by $S1P_2$, ERK5 and CREB, I first analyzed the effect of $S1P_2$ antagonism on CM-dependent ERK5 phosphorylation, and then the effect of both $S1P_2$ and ERK5 inhibition on CREB binding to mpARG II oligonucleotides.

Antagonism of $S1P_2$ abolished CM-mediated ERK5 phosphorylation, suggesting that S1P in CM directly binds to $S1P_2$, provokes MEK5 activation and subsequent ERK5 phosphorylation. Furthermore, authentic S1P was also able to induce ERK5 phosphorylation. In line, $S1P_2$ couples to G_q and G_{12} proteins (Siehler and Manning, 2002), which are known to activate ERK5 (Fukuhara et al., 2000). Antagonizing $S1P_2$ or inhibiting ERK5 also reversed CM-induced CREB binding to mpARG II oligonucledotides, pointing to the importance of $S1P_2$ and ERK5 for its activation. My findings correlate with previous studies showing that S1P induces CREB activation in e.g. vascular smooth muscle (Mathieson and Nixon, 2006) and breast cancer cells (Hadizadeh et al., 2008). In vascular smooth muscle cells CREB activation was proposed to be ERK1/2

mediated because it was prevented by the MEK inhibitors PD98059 and U0126 (Mathieson and Nixon, 2006). Meanwhile, it became evident that these inhibitors, originally assumed to be specific for MEK1/2, also inhibit the MEK5/ERK5 pathway. Thus, there is room for speculation that in vascular smooth muscle S1P also triggers CREB activation by ERK5.

The physiological relevance of $S1P_2$ and ERK signaling was further strengthened by pharmacological inhibition of CM-mediated ARG II up-regulation by JTE013 as well as U0126 in primary peritoneal MΦs from C57BL/6 mice. Although these primary cells already expressed basal levels of ARG II protein in contrast to RAW264.7 MΦs, they still showed a strong increase in ARG II amount upon CM stimulation. A similar observation was made by Louis *et al.*, who noticed basal levels of ARG II mRNA in isolated murine peritoneal MΦs compared to absent expression in RAW264.7 cells. The authors also reported comparable ARG II mRNA induction in both primary and RAW264.7 MΦs in response to LPS (Louis et al., 1998). Both observations demonstrate that, despite basal levels of ARG II protein, murine peritoneal MΦs can engage signaling pathways strongly up-regulating ARG II protein expression.

Thus, my data suggest that CM contributes to a MΦ phenotype switch by enhancing $S1P_2$ signaling, activating ERK5/CREB and subsequently up-regulating ARG II, which modulates NO production in these cells (FIGURE 6.2).

6.1.6 Mechanism of CREB activation by ERK5

Finally, I investigated ERK5-dependent CREB activation in more detail in order to obtain further information on the signaling mechanisms activated in AC-polarized MΦs that are possibly responsible for the regulatory MΦ phenotype.

As proline-directed kinases, ERKs cannot directly phosphorylate CREB at serine 133, whose phosphorylation is a prerequisite for CREB-mediated transcription (Shaywitz and Greenberg, 1999). Instead, they were reported to induce CREB phosphorylation *in vitro* and *in vivo* by activating an intermediate kinase of the RSK family (Watson et al., 2001; Xing et al., 1996). Furthermore, ERK5 can also influence the activation of PKA und subsequently CREB, through the regulation of PDE4D3 and cAMP hydrolysis (Dodge-Kafka et al., 2005).

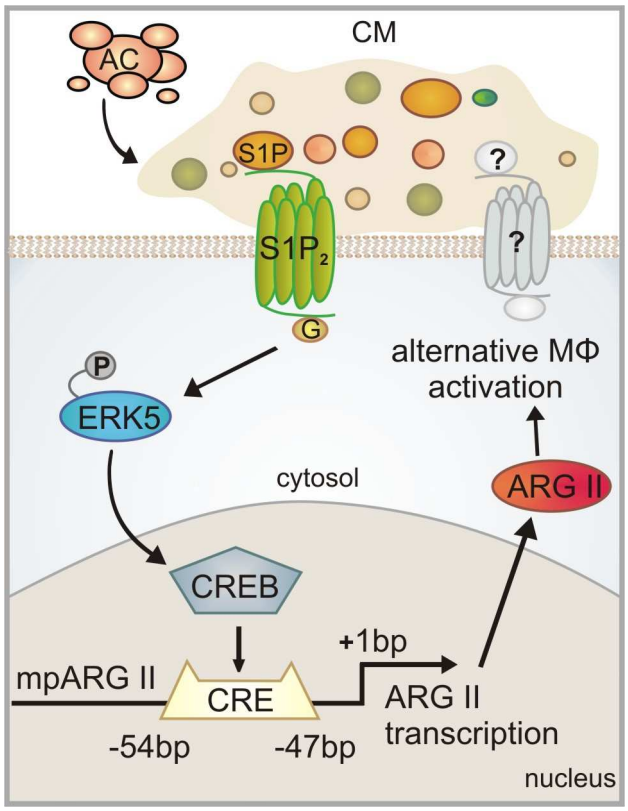

FIGURE 6.2: CM-mediated activation of $S1P_2$, ERK5 and CREB signaling in MΦs. S1P in apoptotic cell (AC)-conditioned medium (CM) contributes to ARG II induction by binding to $S1P_2$ to activate ERK5 and subsequently cyclic adenosine monophosphate responsive element-binding protein (CREB). Besides S1P, an unidentified second factor is required for ARG II up-regulation. ARG II contributes to alternative macrophage (MΦ) activation by impairing nitric oxide (NO) production and by generating the precursor of polyamines.

In my setting the PKA-dependent CREB activation seemed more probable, since ACs have recently been shown to induce cAMP elevation in alveolar MΦs (Medeiros et al., 2009), and cAMP as well as CREB signaling were shown to account for the regulatory phenotype of MΦs (Bystrom et al., 2008; Ruffell et al., 2009).

cAMP was initially considered to be a second messenger able to diffuse freely throughout the cell (Kasai and Petersen, 1994). However, cAMP specifically transduces signals from various receptors and activates different cellular functions. The pleiotropic actions of cAMP thus suggest a tightly regulated localization (Zaccolo and Pozzan, 2002). In fact, accumulating evidence demonstrated gradients in the intracellular distribution of cAMP. These gradients were ensured by compartmentation of cAMP regulators and effectors through the association with scaffold proteins, the AKAPs (Kapiloff, 2002;

Zaccolo et al., 2002). Furthermore, in striated myocytes and neurons, muscle AKAP (mAKAP) was reported to bind cAMP-dependent PKA as well as cAMP-hydrolyzing PDE4D3 in order to spatio-temporally tune cAMP signals. In addition, PDE4D3 served as an adaptor protein for EPAC1 and ERK5. In this setting, ERK5 favored sustained PKA activation by phosphorylating PDE4D3 and thereby decreasing the PDE activity (Dodge-Kafka et al., 2005).

6.1.6.1 Adenylyl cyclase, PKA and PDE4 regulate ARG II expression

Consistant with the observations mentioned above, pharmacological inhibition of adenyly cyclase as well as PKA impaired CM-mediated ARG II up-regulation. The importance of cAMP signaling for ARG II expression was further enforced by forskolin-mediated ARG II induction. The assumption that ERK5 indirectly mediates CREB activation by inhibiting PDE activity was strengthened by using the PDE4 inhibitor rolipram. Indeed, forskolin-dependent activation of adenyly cyclase and subsequent enhanced cAMP levels, in combination with PDE4 inhibition, up-regulated ARG II significantly. Finally, CM-mediated ARG II expression was even stronger when PDE4 was inhibited. Forskolin/rolipram-dependent ARG II induction was strong but still lower compared to CM, indicating that the responsible factors in CM either elevate cAMP or inhibit PDE4 (or both) more efficiently than forskolin/rolipram. The most probable explanation might be the instability of rolipram (Krause et al., 1989), resulting in weaker inhibition of PDE4 compared to CM.

Since I showed that S1P in CM activates $S1P_2$, which has been reported to activate adenylyl cyclase and consequently cAMP production (Spiegel and Milstien, 2003), one might wonder if $S1P_2$ signaling mediates both ERK5 activation and the necessary raise in cAMP levels in my system. However, this hypothesis seems improbable because authentic S1P alone had a minor effect on ARG II protein up-regulation compared to CM.

6.1.6.2 Postulation of an AKAP complex involved in ARG II up-regulation

The implication of PKA, PDE4 and ERK5 in the regulation of ARG II expression suggests a close proximity of these kinases and diesterase in MΦs similar to other systems (see 3.5.1). This could be achieved by an AKAP complex comprising a scaffold protein, PKA, PDE4 and ERK5 thereby regulating cAMP signals in MΦs. A potential candidate might be the scaffold protein AKAP95,

which has recently been shown to play a specific role in the suppression of LPS-dependent TNF-α expression by cAMP. In this context, Wall *et al.* reported that the main anti-inflammatory effects of cAMP in MΦs were specifically mediated by PKA (Wall et al., 2009). Furthermore, the observed CM-dependent time response of cAMP levels also supported the implication of an AKAP complex. The transient raises of cAMP suggested an activation of PDE4 in a negative feedback loop at early time points and the consistent increase in cAMP levels later on pointed to ERK5-mediated inhibition of PDE4. The 1.8 and 1.5-fold induction of cAMP after stimulation with CM for 1 and 10 minutes resp. correlated with an observation made by Medeiros and collegues. They reported a 1.5-fold increase of cAMP in alveolar MΦs incubated with ACs for 30 minutes (Medeiros et al., 2009). The discrepancy in time between the effect of CM and ACs might be explained by the fact that ACs have to secret factors to up-regulate the cAMP production, while these are already present in CM.

6.2 Concluding remarks

In conclusion, my study suggests that a yet unidentified factor in CM provokes an increase of cAMP levels in MΦs inducing PKA activation. As it is known from literature, PKA activity induces a feedback inhibition by enhancing PDE activity resulting in cAMP hydrolysis (Carlisle Michel et al., 2004; Dodge et al., 2001). However, concomitant CM-mediated $S1P_2$ signaling activates ERK5, which probably further enhances PKA activity by inhibiting PDE4. Consequently, sustained CREB activation then provokes steady ARG II up-regulation, which modulates NO production in these cells thereby accounting for regulatory MΦ polarization as well as maintenance (FIGURE 6.3).

Despite the role in alternative MΦ polarization, ARG II has also been tied to tumor biology, as I mentioned before, and to a series of vascular diseases including asthma and hypertension (King et al., 2004; Xu et al., 2004), since these pathophysiological conditions result from arginase-mediated impaired NO production. Although I do not provide any direct evidence, one might wonder if the mechanism described in this study (accounting for ARG II up-regulation) might apply to other systems, like tumor cells or vascular tissue.

I hypothesize that the regulation of CM-mediated cAMP signaling in MΦs is garanteed by a scaffold protein, possibly AKAP95, which tightly holds effectors

and regulators together. Additional experiments are necessary in order to show an actual involvement of a scaffold protein and characterize it. Then, CM-mediated early PDE4 activation followed by ERK5-dependent inhibition should be demonstrated. Finally, co-localization of the single modules of this postulated complex should complete the evidence.

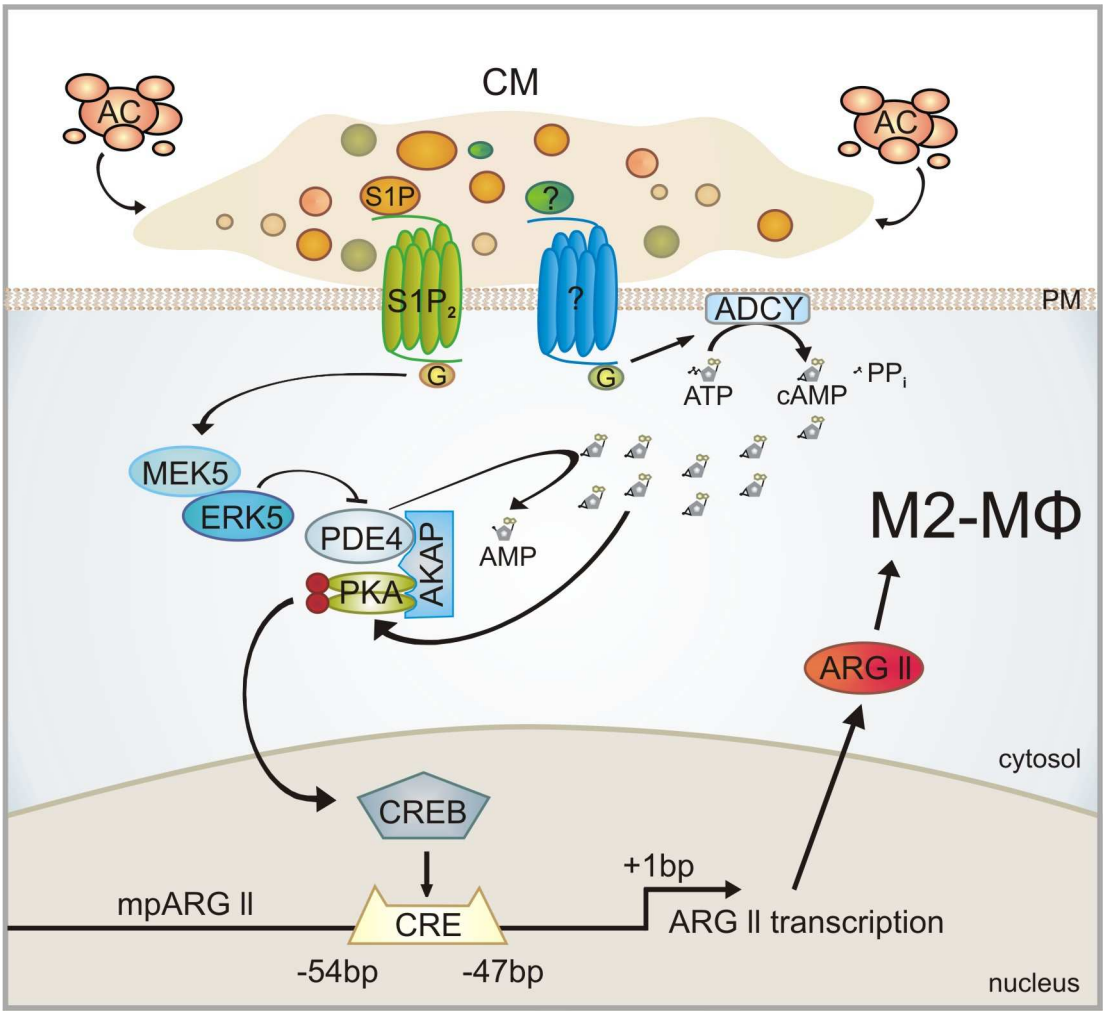

FIGURE 6.3: Summary of ARG II up-regulation by CM.
S1P in apoptotic cell (AC)-conditioned medium (CM) binds to $S1P_2$ to activate ERK5. Another unidentified factor probably binds to its receptor thereby raising cyclic adenosine monophosphate (cAMP) levels. The second messenger cAMP then activates target molecules among which protein kinase A (PKA) is the most prominent. The activated catalytic subunits of PKA phosphorylate/activate cAMP responsive element-binding protein (CREB), which binds to CRE on the promoter region of ARG II (mpARG II), thereby enhancing its expression. ARG II facilitates macrophage (MΦ) M2 polarization by modulating nitric oxide (NO) and polyamine production. PKA is probably hold in close proximity to cAMP-hydrolizing phosphodiesterase 4 (PDE4) by an A kinase-anchoring protein (AKAP). ERK5 might contribute to enhanced cAMP/PKA signaling by inhibiting PDE activity. (PM: plasma membrane, ADCY: adenylyl cyclase, ATP: adenosine triphosphate, PP_i: pyrophosphate, AMP: adenosine monophosphate)

Furthermore, it would be interesting to analyze the impact of ERK5 depletion on MΦ polarization in detail, since this MAPK seems primordial for a sustained cAMP signal in MΦs. A conditional knock-out approach in MΦs would be sensible, in order to circumvent early embryonic lethalty in conventional ERK5 knock-out mice (Moodley et al., 2003). A study in a tumor model with ERK5 conditional knock-out MΦs should provide important insights in the role of the ERK5 signaling cascade in TAMs and the subsequent effects on tumor cells. In this context, the development of a specific pharmacological ERK5 inhibitor might be a sensible approach to treat disorders, where alternative regulatory MΦ activation is detrimental.

7 References

Abel, E. A. (1995). Phototherapy. *Dermatol Clin* **13**, 841-9.

Aderem, A. and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* **17**, 593-623.

Akakura, S., Singh, S., Spataro, M., Akakura, R., Kim, J. I., Albert, M. L. and Birge, R. B. (2004). The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. *Exp Cell Res* **292**, 403-16.

Albert, M. L. (2004). Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nat Rev Immunol* **4**, 223-31.

Anderson, C. F. and Mosser, D. M. (2002). A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J Leukoc Biol* **72**, 101-6.

Baggiolini, M., Walz, A. and Kunkel, S. L. (1989). Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J Clin Invest* **84**, 1045-9.

Balthasar, S., Bergelin, N., Lof, C., Vainio, M., Andersson, S. and Tornquist, K. (2008). Interactions between sphingosine-1-phosphate and vascular endothelial growth factor signalling in ML-1 follicular thyroid carcinoma cells. *Endocr Relat Cancer* **15**, 521-34.

Barksdale, A. R., Bernard, A. C., Maley, M. E., Gellin, G. L., Kearney, P. A., Boulanger, B. R., Tsuei, B. J. and Ochoa, J. B. (2004). Regulation of arginase expression by T-helper II cytokines and isoproterenol. *Surgery* **135**, 527-35.

Benoit, M., Barbarat, B., Bernard, A., Olive, D. and Mege, J. L. (2008). Coxiella burnetii, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. *Eur J Immunol* **38**, 1065-70.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. and Kuchroo, V. K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235-8.

Bingle, L., Brown, N. J. and Lewis, C. E. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* **196**, 254-65.

Biswas, S. K., Gangi, L., Paul, S., Schioppa, T., Saccani, A., Sironi, M., Bottazzi, B., Doni, A., Vincenzo, B., Pasqualini, F. et al. (2006). A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood* **107**, 2112-22.

Bogdan, C. (2001). Nitric oxide and the immune response. *Nat Immunol* **2**, 907-16.

Bogdan, C. and Rollinghoff, M. (1998). The immune response to Leishmania: mechanisms of parasite control and evasion. *Int J Parasitol* **28**, 121-34.

Bondanza, A., Zimmermann, V. S., Rovere-Querini, P., Turnay, J., Dumitriu, I. E., Stach, C. M., Voll, R. E., Gaipl, U. S., Bertling, W., Poschl, E. et al. (2004). Inhibition

of phosphatidylserine recognition heightens the immunogenicity of irradiated lymphoma cells in vivo. *J Exp Med* **200**, 1157-65.

Bonizzi, G. and Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* **25**, 280-8.

Bos, J. L. (2003). Epac: a new cAMP target and new avenues in cAMP research. *Nat Rev Mol Cell Biol* **4**, 733-8.

Bose, J., Gruber, A. D., Helming, L., Schiebe, S., Wegener, I., Hafner, M., Beales, M., Kontgen, F. and Lengeling, A. (2004). The phosphatidylserine receptor has essential functions during embryogenesis but not in apoptotic cell removal. *J Biol* **3**, 15.

Boss, V., Abbott, K. L., Wang, X. F., Pavlath, G. K. and Murphy, T. J. (1998). The cyclosporin A-sensitive nuclear factor of activated T cells (NFAT) proteins are expressed in vascular smooth muscle cells. Differential localization of NFAT isoforms and induction of NFAT-mediated transcription by phospholipase C-coupled cell surface receptors. *J Biol Chem* **273**, 19664-71.

Bournazou, I., Pound, J. D., Duffin, R., Bournazos, S., Melville, L. A., Brown, S. B., Rossi, A. G. and Gregory, C. D. (2009). Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. *J Clin Invest* **119**, 20-32.

Brandt, E., Woerly, G., Younes, A. B., Loiseau, S. and Capron, M. (2000). IL-4 production by human polymorphonuclear neutrophils. *J Leukoc Biol* **68**, 125-30.

Bratton, D. L. and Henson, P. M. (2008). Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? *Curr Biol* **18**, R76-9.

Brouckaert, G., Kalai, M., Krysko, D. V., Saelens, X., Vercammen, D., Ndlovu, M., Haegeman, G., D'Herde, K. and Vandenabeele, P. (2004). Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. *Mol Biol Cell* **15**, 1089-100.

Brown, S., Heinisch, I., Ross, E., Shaw, K., Buckley, C. D. and Savill, J. (2002). Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. *Nature* **418**, 200-3.

Bystrom, J., Evans, I., Newson, J., Stables, M., Toor, I., van Rooijen, N., Crawford, M., Colville-Nash, P., Farrow, S. and Gilroy, D. W. (2008). Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. *Blood* **112**, 4117-27.

Caberoy, N. B., Maiguel, D., Kim, Y. and Li, W. (2009). Identification of tubby and tubby-like protein 1 as eat-me signals by phage display. *Exp Cell Res*.

Carlisle Michel, J. J., Dodge, K. L., Wong, W., Mayer, N. C., Langeberg, L. K. and Scott, J. D. (2004). PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. *Biochem J* **381**, 587-92.

Carnegie, G. K. and Scott, J. D. (2003). A-kinase anchoring proteins and neuronal signaling mechanisms. *Genes Dev* **17**, 1557-68.

Chang, M. K., Bergmark, C., Laurila, A., Horkko, S., Han, K. H., Friedman, P., Dennis, E. A. and Witztum, J. L. (1999). Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited

macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A* **96**, 6353-8.

Chen, W., Frank, M. E., Jin, W. and Wahl, S. M. (2001). TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* **14**, 715-25.

Cikala, M., Alexandrova, O., David, C. N., Proschel, M., Stiening, B., Cramer, P. and Bottger, A. (2004). The phosphatidylserine receptor from Hydra is a nuclear protein with potential Fe(II) dependent oxygenase activity. *BMC Cell Biol* **5**, 26.

Cocca, B. A., Seal, S. N., D'Agnillo, P., Mueller, Y. M., Katsikis, P. D., Rauch, J., Weigert, M. and Radic, M. Z. (2001). Structural basis for autoantibody recognition of phosphatidylserine-beta 2 glycoprotein I and apoptotic cells. *Proc Natl Acad Sci U S A* **98**, 13826-31.

Corbin, J. D., Soderling, T. R. and Park, C. R. (1973). Regulation of adenosine 3',5'-monophosphate-dependent protein kinase. I. Preliminary characterization of the adipose tissue enzyme in crude extracts. *J Biol Chem* **248**, 1813-21.

Cordeiro-da-Silva, A., Tavares, J., Araujo, N., Cerqueira, F., Tomas, A., Kong Thoo Lin, P. and Ouaissi, A. (2004). Immunological alterations induced by polyamine derivatives on murine splenocytes and human mononuclear cells. *Int Immunopharmacol* **4**, 547-56.

Corraliza, I. and Moncada, S. (2002). Increased expression of arginase II in patients with different forms of arthritis. Implications of the regulation of nitric oxide. *J Rheumatol* **29**, 2261-5.

Cvetanovic, M. and Ucker, D. S. (2004). Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol* **172**, 880-9.

Dale, D. C., Boxer, L. and Liles, W. C. (2008). The phagocytes: neutrophils and monocytes. *Blood* **112**, 935-45.

Deshane, J., Wright, M. and Agarwal, A. (2005). Heme oxygenase-1 expression in disease states. *Acta Biochim Pol* **52**, 273-84.

Devitt, A., Moffatt, O. D., Raykundalia, C., Capra, J. D., Simmons, D. L. and Gregory, C. D. (1998). Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* **392**, 505-9.

Devitt, A., Parker, K. G., Ogden, C. A., Oldreive, C., Clay, M. F., Melville, L. A., Bellamy, C. O., Lacy-Hulbert, A., Gangloff, S. C., Goyert, S. M. et al. (2004). Persistence of apoptotic cells without autoimmune disease or inflammation in CD14-/- mice. *J Cell Biol* **167**, 1161-70.

Dodge-Kafka, K. L., Soughayer, J., Pare, G. C., Carlisle Michel, J. J., Langeberg, L. K., Kapiloff, M. S. and Scott, J. D. (2005). The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. *Nature* **437**, 574-8.

Dodge, K. L., Khouangsathiene, S., Kapiloff, M. S., Mouton, R., Hill, E. V., Houslay, M. D., Langeberg, L. K. and Scott, J. D. (2001). mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. *Embo J* **20**, 1921-30.

Edwards, J. P., Zhang, X., Frauwirth, K. A. and Mosser, D. M. (2006). Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol* **80**, 1298-307.

Esparis-Ogando, A., Diaz-Rodriguez, E., Montero, J. C., Yuste, L., Crespo, P. and Pandiella, A. (2002). Erk5 participates in neuregulin signal transduction and is constitutively active in breast cancer cells overexpressing ErbB2. *Mol Cell Biol* **22**, 270-85.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* **101**, 890-8.

Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A. and Henson, P. M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* **405**, 85-90.

Falasca, L., Iadevaia, V., Ciccosanti, F., Melino, G., Serafino, A. and Piacentini, M. (2005). Transglutaminase type II is a key element in the regulation of the anti-inflammatory response elicited by apoptotic cell engulfment. *J Immunol* **174**, 7330-40.

Forman, H. J. and Torres, M. (2002). Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am J Respir Crit Care Med* **166**, S4-8.

Frasch, S. C., Henson, P. M., Kailey, J. M., Richter, D. A., Janes, M. S., Fadok, V. A. and Bratton, D. L. (2000). Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. *J Biol Chem* **275**, 23065-73.

Freire-de-Lima, C. G., Nascimento, D. O., Soares, M. B., Bozza, P. T., Castro-Faria-Neto, H. C., de Mello, F. G., DosReis, G. A. and Lopes, M. F. (2000). Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* **403**, 199-203.

Freire-de-Lima, C. G., Xiao, Y. Q., Gardai, S. J., Bratton, D. L., Schiemann, W. P. and Henson, P. M. (2006). Apoptotic cells, through transforming growth factor-beta, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. *J Biol Chem* **281**, 38376-84.

Fukuhara, S., Marinissen, M. J., Chiariello, M. and Gutkind, J. S. (2000). Signaling from G protein-coupled receptors to ERK5/Big MAPK 1 involves Galpha q and Galpha 12/13 families of heterotrimeric G proteins. Evidence for the existence of a novel Ras AND Rho-independent pathway. *J Biol Chem* **275**, 21730-6.

Gao, Y., Herndon, J. M., Zhang, H., Griffith, T. S. and Ferguson, T. A. (1998). Antiinflammatory effects of CD95 ligand (FasL)-induced apoptosis. *J Exp Med* **188**, 887-96.

Gerber, J. S. and Mosser, D. M. (2001). Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J Immunol* **166**, 6861-8.

Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y. C., Yoshida, M., Ding, H. A., Gimbrone, M. A., Jr., Luster, A. D., Luscinskas, F. W. and Rosenzweig, A. (1999). MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* **398**, 718-23.

Goetzl, E. J., Wang, W., McGiffert, C., Huang, M. C. and Graler, M. H. (2004). Sphingosine 1-phosphate and its G protein-coupled receptors constitute a multifunctional immunoregulatory system. *J Cell Biochem* **92**, 1104-14.

Gomez-Munoz, A., Kong, J., Salh, B. and Steinbrecher, U. P. (2003). Sphingosine-1-phosphate inhibits acid sphingomyelinase and blocks apoptosis in macrophages. *FEBS Lett* **539**, 56-60.

Gordon, S. (2003). Alternative activation of macrophages. *Nat Rev Immunol* **3**, 23-35.

Gotoh, T. and Mori, M. (1999). Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. *J Cell Biol* **144**, 427-34.

Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59-74.

Grandvaux, N., Gaboriau, F., Harris, J., tenOever, B. R., Lin, R. and Hiscott, J. (2005). Regulation of arginase II by interferon regulatory factor 3 and the involvement of polyamines in the antiviral response. *Febs J* **272**, 3120-31.

Grody, W. W., Dizikes, G. J. and Cederbaum, S. D. (1987). Human arginase isozymes. *Isozymes Curr Top Biol Med Res* **13**, 181-214.

Gude, D. R., Alvarez, S. E., Paugh, S. W., Mitra, P., Yu, J., Griffiths, R., Barbour, S. E., Milstien, S. and Spiegel, S. (2008). Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *Faseb J* **22**, 2629-38.

Hadizadeh, S., King, D. N., Shah, S. and Sewer, M. B. (2008). Sphingosine-1-phosphate regulates the expression of the liver receptor homologue-1. *Mol Cell Endocrinol* **283**, 104-13.

Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A. and Nagata, S. (2002). Identification of a factor that links apoptotic cells to phagocytes. *Nature* **417**, 182-7.

Hannun, Y. A. and Obeid, L. M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol* **9**, 139-50.

Hasko, G., Pacher, P., Deitch, E. A. and Vizi, E. S. (2007). Shaping of monocyte and macrophage function by adenosine receptors. *Pharmacol Ther* **113**, 264-75.

Herr, B., Zhou, J., Werno, C., Menrad, H., Namgaladze, D., Weigert, A., Dehne, N. and Brune, B. (2009). The supernatant of apoptotic cells causes transcriptional activation of HIF-1{alpha} in macrophages via sphingosine-1-phosphate and transforming growth factor-{beta}. *Blood*.

Hesse, M., Modolell, M., La Flamme, A. C., Schito, M., Fuentes, J. M., Cheever, A. W., Pearce, E. J. and Wynn, T. A. (2001). Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol* **167**, 6533-44.

Hodge, S., Hodge, G., Flower, R., Reynolds, P. N., Scicchitano, R. and Holmes, M. (2002). Up-regulation of production of TGF-beta and IL-4 and down-regulation of IL-6 by apoptotic human bronchial epithelial cells. *Immunol Cell Biol* **80**, 537-43.

Hotchkiss, R. S. and Karl, I. E. (2003). The pathophysiology and treatment of sepsis. *N Engl J Med* **348**, 138-50.

Houslay, M. D. and Adams, D. R. (2003). PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* **370**, 1-18.

Huang, H., Park, P. H., McMullen, M. R. and Nagy, L. E. (2008). Mechanisms for the anti-inflammatory effects of adiponectin in macrophages. *J Gastroenterol Hepatol* **23 Suppl 1**, S50-3.

Hughes, J., Liu, Y., Van Damme, J. and Savill, J. (1997). Human glomerular mesangial cell phagocytosis of apoptotic neutrophils: mediation by a novel CD36-independent vitronectin receptor/thrombospondin recognition mechanism that is uncoupled from chemokine secretion. *J Immunol* **158**, 4389-97.

Hughes, J. E., Srinivasan, S., Lynch, K. R., Proia, R. L., Ferdek, P. and Hedrick, C. C. (2008). Sphingosine-1-phosphate induces an antiinflammatory phenotype in macrophages. *Circ Res* **102**, 950-8.

Huynh, M. L., Fadok, V. A. and Henson, P. M. (2002). Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* **109**, 41-50.

Idzko, M., Panther, E., Corinti, S., Morelli, A., Ferrari, D., Herouy, Y., Dichmann, S., Mockenhaupt, M., Gebicke-Haerter, P., Di Virgilio, F. et al. (2002). Sphingosine 1-phosphate induces chemotaxis of immature and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses. *Faseb J* **16**, 625-7.

Igarashi, Y., Kitamura, K., Toyokuni, T., Dean, B., Fenderson, B., Ogawass, T. and Hakomori, S. (1990). A specific enhancing effect of N,N-dimethylsphingosine on epidermal growth factor receptor autophosphorylation. Demonstration of its endogenous occurrence (and the virtual absence of unsubstituted sphingosine) in human epidermoid carcinoma A431 cells. *J Biol Chem* **265**, 5385-9.

Jennewein, C., Kuhn, A. M., Schmidt, M. V., Meilladec-Jullig, V., von Knethen, A., Gonzalez, F. J. and Brune, B. (2008). Sumoylation of peroxisome proliferator-activated receptor gamma by apoptotic cells prevents lipopolysaccharide-induced NCoR removal from kappaB binding sites mediating transrepression of proinflammatory cytokines. *J Immunol* **181**, 5646-52.

Jiang, L. I., Collins, J., Davis, R., Lin, K. M., DeCamp, D., Roach, T., Hsueh, R., Rebres, R. A., Ross, E. M., Taussig, R. et al. (2007). Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J Biol Chem* **282**, 10576-84.

Johann, A. M., Barra, V., Kuhn, A. M., Weigert, A., von Knethen, A. and Brune, B. (2007). Apoptotic cells induce arginase II in macrophages, thereby attenuating NO production. *Faseb J* **21**, 2704-12.

Johann, A. M., von Knethen, A., Lindemann, D. and Brune, B. (2006). Recognition of apoptotic cells by macrophages activates the peroxisome proliferator-activated receptor-gamma and attenuates the oxidative burst. *Cell Death Differ* **13**, 1533-40.

Kagan, V. E., Borisenko, G. G., Serinkan, B. F., Tyurina, Y. Y., Tyurin, V. A., Jiang, J., Liu, S. X., Shvedova, A. A., Fabisiak, J. P., Uthaisang, W. et al. (2003).

Appetizing rancidity of apoptotic cells for macrophages: oxidation, externalization, and recognition of phosphatidylserine. *Am J Physiol Lung Cell Mol Physiol* **285**, L1-17.

Kagan, V. E., Gleiss, B., Tyurina, Y. Y., Tyurin, V. A., Elenstrom-Magnusson, C., Liu, S. X., Serinkan, F. B., Arroyo, A., Chandra, J., Orrenius, S. et al. (2002). A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J Immunol* **169**, 487-99.

Kapiloff, M. S. (2002). Contributions of protein kinase A anchoring proteins to compartmentation of cAMP signaling in the heart. *Mol Pharmacol* **62**, 193-9.

Kasai, H. and Petersen, O. H. (1994). Spatial dynamics of second messengers: IP3 and cAMP as long-range and associative messengers. *Trends Neurosci* **17**, 95-101.

Kaupp, U. B. and Seifert, R. (2002). Cyclic nucleotide-gated ion channels. *Physiol Rev* **82**, 769-824.

Kerr, J. F. (1965). A histochemical study of hypertrophy and ischaemic injury of rat liver with special reference to changes in lysosomes. *J Pathol Bacteriol* **90**, 419-35.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-57.

Kim, C., Wilcox-Adelman, S., Sano, Y., Tang, W. J., Collier, R. J. and Park, J. M. (2008). Antiinflammatory cAMP signaling and cell migration genes co-opted by the anthrax bacillus. *Proc Natl Acad Sci U S A* **105**, 6150-5.

Kim, H. P., Ryter, S. W. and Choi, A. M. (2006). CO as a cellular signaling molecule. *Annu Rev Pharmacol Toxicol* **46**, 411-49.

King, N. E., Rothenberg, M. E. and Zimmermann, N. (2004). Arginine in asthma and lung inflammation. *J Nutr* **134**, 2830S-2836S; discussion 2853S.

Kluk, M. J. and Hla, T. (2002). Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochim Biophys Acta* **1582**, 72-80.

Knowles, H. J. and Harris, A. L. (2001). Hypoxia and oxidative stress in breast cancer. Hypoxia and tumourigenesis. *Breast Cancer Res* **3**, 318-22.

Krause, W., Kuhne, G. and Matthes, H. (1989). Pharmacokinetics of the antidepressant rolipram in healthy volunteers. *Xenobiotica* **19**, 683-92.

Krysko, D. V., Brouckaert, G., Kalai, M., Vandenabeele, P. and D'Herde, K. (2003). Mechanisms of internalization of apoptotic and necrotic L929 cells by a macrophage cell line studied by electron microscopy. *J Morphol* **258**, 336-45.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-74.

Lambeth, J. D. (2004). NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* **4**, 181-9.

Larsen, E. C., DiGennaro, J. A., Saito, N., Mehta, S., Loegering, D. J., Mazurkiewicz, J. E. and Lennartz, M. R. (2000). Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J Immunol* **165**, 2809-17.

Lauber, K., Blumenthal, S. G., Waibel, M. and Wesselborg, S. (2004). Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell* **14**, 277-87.

Lauber, K., Bohn, E., Krober, S. M., Xiao, Y. J., Blumenthal, S. G., Lindemann, R. K., Marini, P., Wiedig, C., Zobywalski, A., Baksh, S. et al. (2003). Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* **113**, 717-30.

Le Stunff, H., Milstien, S. and Spiegel, S. (2004). Generation and metabolism of bioactive sphingosine-1-phosphate. *J Cell Biochem* **92**, 882-99.

Lemke, G. and Rothlin, C. V. (2008). Immunobiology of the TAM receptors. *Nat Rev Immunol* **8**, 327-36.

Levitzki, A. (1988). From epinephrine to cyclic AMP. *Science* **241**, 800-6.

Lewis, C. E. and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66**, 605-12.

Lin, E. Y., Li, J. F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., Qian, H., Xue, X. N. and Pollard, J. W. (2006). Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res* **66**, 11238-46.

Liu, C. H., Chang, S. H., Narko, K., Trifan, O. C., Wu, M. T., Smith, E., Haudenschild, C., Lane, T. F. and Hla, T. (2001). Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *J Biol Chem* **276**, 18563-9.

Loke, P., Gallagher, I., Nair, M. G., Zang, X., Brombacher, F., Mohrs, M., Allison, J. P. and Allen, J. E. (2007). Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *J Immunol* **179**, 3926-36.

Louis, C. A., Reichner, J. S., Henry, W. L., Jr., Mastrofrancesco, B., Gotoh, T., Mori, M. and Albina, J. E. (1998). Distinct arginase isoforms expressed in primary and transformed macrophages: regulation by oxygen tension. *Am J Physiol* **274**, R775-82.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265-75.

Lucas, M., Stuart, L. M., Zhang, A., Hodivala-Dilke, K., Febbraio, M., Silverstein, R., Savill, J. and Lacy-Hulbert, A. (2006). Requirements for apoptotic cell contact in regulation of macrophage responses. *J Immunol* **177**, 4047-54.

Lucas, M., Zhang, X., Prasanna, V. and Mosser, D. M. (2005). ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus. *J Immunol* **175**, 469-77.

Mackaness, G. B. (1964). The Immunological Basis of Acquired Cellular Resistance. *J Exp Med* **120**, 105-20.

Mantovani, A., Allavena, P. and Sica, A. (2004a). Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. *Eur J Cancer* **40**, 1660-7.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. (2004b). The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* **25**, 677-86.

Marathe, C., Bradley, M. N., Hong, C., Lopez, F., Ruiz de Galarreta, C. M., Tontonoz, P. and Castrillo, A. (2006). The arginase II gene is an anti-inflammatory target of liver X receptor in macrophages. *J Biol Chem* **281**, 32197-206.

Martinez, F. O., Sica, A., Mantovani, A. and Locati, M. (2008). Macrophage activation and polarization. *Front Biosci* **13**, 453-61.

Mathieson, F. A. and Nixon, G. F. (2006). Sphingolipids differentially regulate mitogen-activated protein kinases and intracellular Ca2+ in vascular smooth muscle: effects on CREB activation. *Br J Pharmacol* **147**, 351-9.

McCracken, S. R., Ramsay, A., Heer, R., Mathers, M. E., Jenkins, B. L., Edwards, J., Robson, C. N., Marquez, R., Cohen, P. and Leung, H. Y. (2008). Aberrant expression of extracellular signal-regulated kinase 5 in human prostate cancer. *Oncogene* **27**, 2978-88.

Medeiros, A. I., Serezani, C. H., Lee, S. P. and Peters-Golden, M. (2009). Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE2/EP2 signaling. *J Exp Med* **206**, 61-8.

Mevorach, D., Mascarenhas, J. O., Gershov, D. and Elkon, K. B. (1998). Complement-dependent clearance of apoptotic cells by human macrophages. *J Exp Med* **188**, 2313-20.

Meyer zu Heringdorf, D., Lass, H., Kuchar, I., Lipinski, M., Alemany, R., Rumenapp, U. and Jakobs, K. H. (2001). Stimulation of intracellular sphingosine-1-phosphate production by G-protein-coupled sphingosine-1-phosphate receptors. *Eur J Pharmacol* **414**, 145-54.

Mitchell, J. E., Cvetanovic, M., Tibrewal, N., Patel, V., Colamonici, O. R., Li, M. O., Flavell, R. A., Levine, J. S., Birge, R. B. and Ucker, D. S. (2006). The presumptive phosphatidylserine receptor is dispensable for innate anti-inflammatory recognition and clearance of apoptotic cells. *J Biol Chem* **281**, 5718-25.

Mitra, P., Oskeritzian, C. A., Payne, S. G., Beaven, M. A., Milstien, S. and Spiegel, S. (2006). Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc Natl Acad Sci U S A* **103**, 16394-9.

Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T. and Nagata, S. (2007). Identification of Tim4 as a phosphatidylserine receptor. *Nature* **450**, 435-9.

Moodley, Y., Rigby, P., Bundell, C., Bunt, S., Hayashi, H., Misso, N., McAnulty, R., Laurent, G., Scaffidi, A., Thompson, P. et al. (2003). Macrophage recognition and phagocytosis of apoptotic fibroblasts is critically dependent on fibroblast-derived thrombospondin 1 and CD36. *Am J Pathol* **162**, 771-9.

Morimoto, H., Kondoh, K., Nishimoto, S., Terasawa, K. and Nishida, E. (2007). Activation of a C-terminal transcriptional activation domain of ERK5 by autophosphorylation. *J Biol Chem* **282**, 35449-56.

Morris, S. M., Jr. (2004). Enzymes of arginine metabolism. *J Nutr* **134**, 2743S-2747S; discussion 2765S-2767S.

Morris, S. M., Jr., Kepka-Lenhart, D. and Chen, L. C. (1998). Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. *Am J Physiol* **275**, E740-7.

Mosser, D. M. (2003). The many faces of macrophage activation. *J Leukoc Biol* **73**, 209-12.

Mosser, D. M. and Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **8**, 958-69.

Mukundan, L., Odegaard, J. I., Morel, C. R., Heredia, J. E., Mwangi, J. W., Ricardo-Gonzalez, R. R., Goh, Y. P., Eagle, A. R., Dunn, S. E., Awakuni, J. U. et al. (2009). PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance. *Nat Med* **15**, 1266-72.

Mumenthaler, S. M., Yu, H., Tze, S., Cederbaum, S. D., Pegg, A. E., Seligson, D. B. and Grody, W. W. (2008). Expression of arginase II in prostate cancer. *Int J Oncol* **32**, 357-65.

Munitz, A., Brandt, E. B., Mingler, M., Finkelman, F. D. and Rothenberg, M. E. (2008). Distinct roles for IL-13 and IL-4 via IL-13 receptor alpha1 and the type II IL-4 receptor in asthma pathogenesis. *Proc Natl Acad Sci U S A* **105**, 7240-5.

Murdoch, C., Giannoudis, A. and Lewis, C. E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* **104**, 2224-34.

O'Shea, J. J. and Murray, P. J. (2008). Cytokine signaling modules in inflammatory responses. *Immunity* **28**, 477-87.

Ogden, C. A., deCathelineau, A., Hoffmann, P. R., Bratton, D., Ghebrehiwet, B., Fadok, V. A. and Henson, P. M. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* **194**, 781-95.

Otterbein, L. E., Bach, F. H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R. J., Flavell, R. A. and Choi, A. M. (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* **6**, 422-8.

Park, D., Tosello-Trampont, A. C., Elliott, M. R., Lu, M., Haney, L. B., Ma, Z., Klibanov, A. L., Mandell, J. W. and Ravichandran, K. S. (2007). BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature* **450**, 430-4.

Park, S. Y., Jung, M. Y., Kim, H. J., Lee, S. J., Kim, S. Y., Lee, B. H., Kwon, T. H., Park, R. W. and Kim, I. S. (2008). Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death Differ* **15**, 192-201.

Pascual, G., Fong, A. L., Ogawa, S., Gamliel, A., Li, A. C., Perissi, V., Rose, D. W., Willson, T. M., Rosenfeld, M. G. and Glass, C. K. (2005). A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759-63.

Peter, C., Waibel, M., Radu, C. G., Yang, L. V., Witte, O. N., Schulze-Osthoff, K., Wesselborg, S. and Lauber, K. (2008). Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *J Biol Chem* **283**, 5296-305.

Pettus, B. J., Bielawska, A., Subramanian, P., Wijesinghe, D. S., Maceyka, M., Leslie, C. C., Evans, J. H., Freiberg, J., Roddy, P., Hannun, Y. A. et al. (2004). Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. *J Biol Chem* **279**, 11320-6.

Pierce, K. L., Premont, R. T. and Lefkowitz, R. J. (2002). Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**, 639-50.

Platt, N., Suzuki, H., Kodama, T. and Gordon, S. (2000). Apoptotic thymocyte clearance in scavenger receptor class A-deficient mice is apparently normal. *J Immunol* **164**, 4861-7.

Pollard, J. W. (2008). Macrophages define the invasive microenvironment in breast cancer. *J Leukoc Biol* **84**, 623-30.

Porembska, Z., Luboinski, G., Chrzanowska, A., Mielczarek, M., Magnuska, J. and Baranczyk-Kuzma, A. (2003). Arginase in patients with breast cancer. *Clin Chim Acta* **328**, 105-11.

Potter, R. L. and Taylor, S. S. (1979). Relationships between structural domains and function in the regulatory subunit of cAMP-dependent protein kinases I and II from porcine skeletal muscle. *J Biol Chem* **254**, 2413-8.

Raschke, W. C., Baird, S., Ralph, P. and Nakoinz, I. (1978). Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* **15**, 261-7.

Reiter, I., Krammer, B. and Schwamberger, G. (1999). Cutting edge: differential effect of apoptotic versus necrotic tumor cells on macrophage antitumor activities. *J Immunol* **163**, 1730-2.

Ren, Y., Silverstein, R. L., Allen, J. and Savill, J. (1995). CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis. *J Exp Med* **181**, 1857-62.

Romieu-Mourez, R., Solis, M., Nardin, A., Goubau, D., Baron-Bodo, V., Lin, R., Massie, B., Salcedo, M. and Hiscott, J. (2006). Distinct roles for IFN regulatory factor (IRF)-3 and IRF-7 in the activation of antitumor properties of human macrophages. *Cancer Res* **66**, 10576-85.

Rotondo, R., Mastracci, L., Piazza, T., Barisione, G., Fabbi, M., Cassanello, M., Costa, R., Morandi, B., Astigiano, S., Cesario, A. et al. (2008). Arginase 2 is expressed by human lung cancer, but it neither induces immune suppression, nor affects disease progression. *Int J Cancer* **123**, 1108-16.

Rovida, E., Spinelli, E., Sdelci, S., Barbetti, V., Morandi, A., Giuntoli, S. and Dello Sbarba, P. (2008). ERK5/BMK1 is indispensable for optimal colony-stimulating factor 1 (CSF-1)-induced proliferation in macrophages in a Src-dependent fashion. *J Immunol* **180**, 4166-72.

Ruffell, D., Mourkioti, F., Gambardella, A., Kirstetter, P., Lopez, R. G., Rosenthal, N. and Nerlov, C. (2009). A CREB-C/EBP{beta} cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A*.

Saccani, A., Schioppa, T., Porta, C., Biswas, S. K., Nebuloni, M., Vago, L., Bottazzi, B., Colombo, M. P., Mantovani, A. and Sica, A. (2006). p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res* **66**, 11432-40.

Savill, J., Dransfield, I., Gregory, C. and Haslett, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol* **2**, 965-75.

Savill, J., Dransfield, I., Hogg, N. and Haslett, C. (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature* **343**, 170-3.

Savill, J. and Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature* **407**, 784-8.

Savill, J., Fadok, V., Henson, P. and Haslett, C. (1993). Phagocyte recognition of cells undergoing apoptosis. *Immunol Today* **14**, 131-6.

Savill, J., Hogg, N., Ren, Y. and Haslett, C. (1992). Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest* **90**, 1513-22.

Scapoli, L., Ramos-Nino, M. E., Martinelli, M. and Mossman, B. T. (2004). Src-dependent ERK5 and Src/EGFR-dependent ERK1/2 activation is required for cell proliferation by asbestos. *Oncogene* **23**, 805-13.

Schagat, T. L., Wofford, J. A. and Wright, J. R. (2001). Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils. *J Immunol* **166**, 2727-33.

Schneider, U. and Schwenk, H. U. (1977). Characterization of "T" and "non-T" cell lines established from children with acute lymphoblastic leukemia and non-Hodgkin lymphoma after leukemic transformation. *Haematol Blood Transfus* **20**, 265-9.

Sen, P., Wallet, M. A., Yi, Z., Huang, Y., Henderson, M., Mathews, C. E., Earp, H. S., Matsushima, G., Baldwin, A. S., Jr. and Tisch, R. M. (2007). Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. *Blood* **109**, 653-60.

Serinkan, B. F., Gambelli, F., Potapovich, A. I., Babu, H., Di Giuseppe, M., Ortiz, L. A., Fabisiak, J. P. and Kagan, V. E. (2005). Apoptotic cells quench reactive oxygen and nitrogen species and modulate TNF-alpha/TGF-beta1 balance in activated macrophages: involvement of phosphatidylserine-dependent and -independent pathways. *Cell Death Differ* **12**, 1141-4.

Sharma, G. and Goalstone, M. L. (2005). Dominant negative FTase (DNFTalpha) inhibits ERK5, MEF2C and CREB activation in adipogenesis. *Mol Cell Endocrinol* **245**, 93-104.

Shaywitz, A. J. and Greenberg, M. E. (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* **68**, 821-61.

Siehler, S. and Manning, D. R. (2002). Pathways of transduction engaged by sphingosine 1-phosphate through G protein-coupled receptors. *Biochim Biophys Acta* **1582**, 94-9.

Singh, R., Pervin, S., Karimi, A., Cederbaum, S. and Chaudhuri, G. (2000). Arginase activity in human breast cancer cell lines: N(omega)-hydroxy-L-arginine selectively inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells. *Cancer Res* **60**, 3305-12.

Soule, H. D., Vazguez, J., Long, A., Albert, S. and Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* **51**, 1409-16.

Spiegel, S. and Milstien, S. (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4**, 397-407.

Stein, M., Keshav, S., Harris, N. and Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* **176**, 287-92.

Stern, M., Savill, J. and Haslett, C. (1996). Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha v beta 3/CD36/thrombospondin recognition mechanism and lack of phlogistic response. *Am J Pathol* **149**, 911-21.

Stowell, S. R., Karmakar, S., Arthur, C. M., Ju, T., Rodrigues, L. C., Riul, T. B., Dias-Baruffi, M., Miner, J., McEver, R. P. and Cummings, R. D. (2009). Galectin-1 induces reversible phosphatidylserine exposure at the plasma membrane. *Mol Biol Cell* **20**, 1408-18.

Strassmann, G., Patil-Koota, V., Finkelman, F., Fong, M. and Kambayashi, T. (1994). Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med* **180**, 2365-70.

Stuart, L. M. and Ezekowitz, R. A. (2005). Phagocytosis: elegant complexity. *Immunity* **22**, 539-50.

Swann, J. B., Vesely, M. D., Silva, A., Sharkey, J., Akira, S., Schreiber, R. D. and Smyth, M. J. (2008). Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. *Proc Natl Acad Sci U S A* **105**, 652-6.

Szekanecz, Z. and Koch, A. E. (2007). Macrophages and their products in rheumatoid arthritis. *Curr Opin Rheumatol* **19**, 289-95.

Tate, D. J., Jr., Vonderhaar, D. J., Caldas, Y. A., Metoyer, T., Patterson, J. R. t., Aviles, D. H. and Zea, A. H. (2008). Effect of arginase II on L-arginine depletion and cell growth in murine cell lines of renal cell carcinoma. *J Hematol Oncol* **1**, 14.

Taylor, P. R., Carugati, A., Fadok, V. A., Cook, H. T., Andrews, M., Carroll, M. C., Savill, J. S., Henson, P. M., Botto, M. and Walport, M. J. (2000). A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J Exp Med* **192**, 359-66.

Tibrewal, N., Wu, Y., D'Mello, V., Akakura, R., George, T. C., Varnum, B. and Birge, R. B. (2008). Autophosphorylation docking site Tyr-867 in Mer receptor tyrosine kinase allows for dissociation of multiple signaling pathways for phagocytosis of apoptotic cells and down-modulation of lipopolysaccharide-inducible NF-kappaB transcriptional activation. *J Biol Chem* **283**, 3618-27.

Topal, G., Brunet, A., Walch, L., Boucher, J. L. and David-Dufilho, M. (2006). Mitochondrial arginase II modulates nitric-oxide synthesis through nonfreely exchangeable L-arginine pools in human endothelial cells. *J Pharmacol Exp Ther* **318**, 1368-74.

Venkataraman, K., Thangada, S., Michaud, J., Oo, M. L., Ai, Y., Lee, Y. M., Wu, M., Parikh, N. S., Khan, F., Proia, R. L. et al. (2006). Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. *Biochem J* **397**, 461-71.

Verhoven, B., Schlegel, R. A. and Williamson, P. (1995). Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J Exp Med* **182**, 1597-601.

Vockley, J. G., Jenkinson, C. P., Shukla, H., Kern, R. M., Grody, W. W. and Cederbaum, S. D. (1996). Cloning and characterization of the human type II arginase gene. *Genomics* **38**, 118-23.

Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R. and Girkontaite, I. (1997). Immunosuppressive effects of apoptotic cells. *Nature* **390**, 350-1.

von Knethen, A., Lotero, A. and Brune, B. (1998). Etoposide and cisplatin induced apoptosis in activated RAW 264.7 macrophages is attenuated by cAMP-induced gene expression. *Oncogene* **17**, 387-94.

von Knethen, A., Soller, M., Tzieply, N., Weigert, A., Johann, A. M., Jennewein, C., Kohl, R. and Brune, B. (2007). PPARgamma1 attenuates cytosol to membrane translocation of PKCalpha to desensitize monocytes/macrophages. *J Cell Biol* **176**, 681-94.

Von Knethen, A. A. and Brune, B. (2001). Delayed activation of PPARgamma by LPS and IFN-gamma attenuates the oxidative burst in macrophages. *Faseb J* **15**, 535-44.

Wakasugi, K. and Schimmel, P. (1999). Two distinct cytokines released from a human aminoacyl-tRNA synthetase. *Science* **284**, 147-51.

Wall, E. A., Zavzavadjian, J. R., Chang, M. S., Randhawa, B., Zhu, X., Hsueh, R. C., Liu, J., Driver, A., Bao, X. R., Sternweis, P. C. et al. (2009). Suppression of LPS-induced TNF-alpha production in macrophages by cAMP is mediated by PKA-AKAP95-p105. *Sci Signal* **2**, ra28.

Walsh, D. A., Perkins, J. P. and Krebs, E. G. (1968). An adenosine 3',5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. *J Biol Chem* **243**, 3763-5.

Watson, F. L., Heerssen, H. M., Bhattacharyya, A., Klesse, L., Lin, M. Z. and Segal, R. A. (2001). Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. *Nat Neurosci* **4**, 981-8.

Weigert, A., Johann, A. M., von Knethen, A., Schmidt, H., Geisslinger, G. and Brune, B. (2006). Apoptotic cells promote macrophage survival by releasing the antiapoptotic mediator sphingosine-1-phosphate. *Blood* **108**, 1635-42.

Weigert, A., Tzieply, N., von Knethen, A., Johann, A. M., Schmidt, H., Geisslinger, G. and Brune, B. (2007). Tumor cell apoptosis polarizes macrophages role of sphingosine-1-phosphate. *Mol Biol Cell* **18**, 3810-9.

Weis, N., Weigert, A., von Knethen, A. and Brune, B. (2009). Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. *Mol Biol Cell* **20**, 1280-8.

Woo, C. H., Massett, M. P., Shishido, T., Itoh, S., Ding, B., McClain, C., Che, W., Vulapalli, S. R., Yan, C. and Abe, J. (2006). ERK5 activation inhibits inflammatory responses via peroxisome proliferator-activated receptor delta (PPARdelta) stimulation. *J Biol Chem* **281**, 32164-74.

Wu, Y., Tibrewal, N. and Birge, R. B. (2006). Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol* **16**, 189-97.

Wyllie, A. H., Kerr, J. F. and Currie, A. R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* **68**, 251-306.

Xiao, Y. Q., Freire-de-Lima, C. G., Schiemann, W. P., Bratton, D. L., Vandivier, R. W. and Henson, P. M. (2008). Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *J Immunol* **181**, 3575-85.

Xiao, Y. Q., Malcolm, K., Worthen, G. S., Gardai, S., Schiemann, W. P., Fadok, V. A., Bratton, D. L. and Henson, P. M. (2002). Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. *J Biol Chem* **277**, 14884-93.

Xin, C., Ren, S., Kleuser, B., Shabahang, S., Eberhardt, W., Radeke, H., Schafer-Korting, M., Pfeilschifter, J. and Huwiler, A. (2004). Sphingosine 1-phosphate cross-activates the Smad signaling cascade and mimics transforming growth factor-beta-induced cell responses. *J Biol Chem* **279**, 35255-62.

Xing, J., Ginty, D. D. and Greenberg, M. E. (1996). Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**, 959-63.

Xu, W., Kaneko, F. T., Zheng, S., Comhair, S. A., Janocha, A. J., Goggans, T., Thunnissen, F. B., Farver, C., Hazen, S. L., Jennings, C. et al. (2004). Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *Faseb J* **18**, 1746-8.

Xu, Y., Xiao, Y. J., Zhu, K., Baudhuin, L. M., Lu, J., Hong, G., Kim, K. S., Cristina, K. L., Song, L., F, S. W. et al. (2003). Unfolding the pathophysiological role of bioactive lysophospholipids. *Curr Drug Targets Immune Endocr Metabol Disord* **3**, 23-32.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. et al. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301**, 640-3.

Yu, J. L. and Rak, J. W. (2003). Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. *Breast Cancer Res* **5**, 83-8.

Zaccolo, M., Magalhaes, P. and Pozzan, T. (2002). Compartmentalisation of cAMP and Ca(2+) signals. *Curr Opin Cell Biol* **14**, 160-6.

Zaccolo, M. and Pozzan, T. (2002). Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* **295**, 1711-5.

Zhang, X. and Mosser, D. M. (2008). Macrophage activation by endogenous danger signals. *J Pathol* **214**, 161-78.

Zhu, W., Downey, J. S., Gu, J., Di Padova, F., Gram, H. and Han, J. (2000). Regulation of TNF expression by multiple mitogen-activated protein kinase pathways. *J Immunol* **164**, 6349-58

8 Appendices

8.1 Buffers and solutions

8.1.1 Buffer for cell biology

Phosphate buffered saline (PBS):

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	8.1 mM
KH_2PO_4	1.5 mM

→ Adjust pH to 7.4

8.1.2 Buffers and solutions for protein analysis

Hypotonic cell lysis buffer:

HEPES	10 mM	Freshly added prior to use:	
$MgCl_2$	2 mM	DTT	1 mM
EDTA	100 µM	PMSF	0.5 mM
KCl	10 mM	Protease inhibitor mix	1 x

→ Adjust pH to 7.9

Nuclear lysis buffer:

HEPES	50 mM	Freshly added prior to use:	
KCl	50 mM	DTT	1 mM
NaCl	300 mM	PMSF	0.5 mM
EDTA	100 µM	Protease inhibitor mix	1 x
Glycerol	10% (v/v)		

→ Adjust pH to 7.9

Protein lysis buffer

Tris/HCl	50 mM	Freshly added prior to use:	
EDTA	5 mM	PMSF	0.5 mM
NaCl	150 mM	DTT	1 mM
Nonidet P-40	0.5% (v/v)	Protease inhibitor mix	1 x

→ Adjust pH to 8.0

SDS sample buffer (4 x)

Tris/HCl	125 mM
SDS	2% (v/v)
Glycerol	20% (v/v)
Bromophenol blue	0.002% (w/v)
DTT	5 mM

→ Adjust pH to 6.9

SDS-running buffer

Tris/HCl	25 mM
Glycine	192 mM
SDS	0.7 mM

→ Adjust pH to 8.3

Upper tris buffer (4 x)

Tris/HCl	0.5 M

→ Adjust pH to 6.8

Lower tris buffer (4 x)

Tris/HCl	1.5 M

→ Adjust pH to 8.8

Sodium dodecyl sulfate (SDS)-polyacrylamide gels

	Seperating gels 10%	Stacking gel 4%
40% Acrylamide/Bis-acrylamide (37.5% : 1.0% w/v)	2.5 ml	300 µl
Lower tris buffer (4 x)	2.5 ml	
Upper tris buffer (4 x)		750 µl
dH_2O	4.9 ml	1.95 ml
10% SDS	100 µl	30 µl
TEMED	10 µl	2.5 µl
10% (w/v) ammonium persulfate	100 µl	25 µl

Blotting buffer:

Tris-HCl	25 mM
Glycine	192 mM
Methanol	20% (v/v)

→ Check pH to be 8.3

TBS (tris buffered saline)

Tris/HCl	50 mM
NaCl	140 mM

→ Adjust pH to 7.4

TTBS

Tween-20	0.06% (v/v)

→ in TBS

8.1.3 Buffers for molecular biology and microbiology

SOC medium

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	2 mM

EMSA running buffer

Running buffer (glycerol tolerant)

(Purchased from Amersham Biosciences Europe GmbH, Freiburg, Germany)

Native acrylamide gels

	Separating gels 4%
40% Acrylamide/Bis-acrylamide (29% : 1.0% w/v)	1 ml
1 x glycerol tolerant	0.5 ml
dH_2O	8.5 ml

TEMED	32 µl
10% (w/v) ammonium persulfate	100 µl

8.1.4 Buffers for luciferase assay

Luciferase lysis buffer

Tris-H_3PO_4	125 mM
DTT	10 mM
Triton X 100	5%
Glycerol	50%

→ Adjust pH to 7.8

Luciferase assay reagent

Tricine	20 mM
$(MgCO_3)_4$ x $Mg(OH)_2$ x 5 H_2O	1.07 mM
$MgSO_4$ x 7 H_2O	2.67 mM
EDTA-K^+	100 µM
DTT	33.3 mM
ATP	530 µM
Coenzyme A lithium	0.213 mg/ml
D-luciferine	470 mM

→ Check pH to be 7.8

9 Publications

Articles

Cellular and Molecular Life Sciences. 2010 [in revision]

Apoptotic cell-derived factors induce arginase II expression in murine macrophages by activating ERK5/CREB.

Barra V, von Knethen A, Weigert A, Brüne B

J Immunol. 2008; 180(2):1239-48

Apoptotic cell-derived sphingosine-1-phosphate promotes HuR-dependent cyclooxygenase-2 mRNA stabilization and protein expression.

Johann AM, Weigert A, Eberhardt W, Kuhn AM, Barra V, von Knethen A, Pfeilschifter JM, Brüne B

FASEB J.2007; 21(11):2704-12

Apoptotic cells induce arginase II in macrophages, thereby attenuating NO production.

Johann AM, Barra V, Kuhn AM, Weigert A, von Knethen A, Brüne B

Poster presentations

06.-09.09.2006	16th European Congress of Immunology, Paris (France)
18.-20.09.2008	22nd Annual Meeting of EMDS: Diversity and Plasticity of the Innate Immune Response, Brescia (Italy)
15.05.2009	Symposium of the Immunology Department CRP-Santé LNS, Luxemburg (Luxemburg)
27.-30.01.2010	Inflammation 2010: Inflammatory cell signaling mechanisms as therapeutic targets, Luxemburg (Luxemburg)

Scholarship

18.-20.09.2008	22nd Annual Meeting of EMDS: Young Investigator EMDS Conference Travel Award

10 Danksagung

Die vorliegende Doktorarbeit wurde am Institut für Biochemie I - Pathobiochemie der Johann Wolfgang Goethe Universität Frankfurt am Main unter der Leitung von Herrn Prof. Dr. Bernhard Brüne durchgeführt. Bei ihm möchte ich mich für die Bereitstellung des Themas und der benötigten Hilfsmittel, vor allem aber für die ständige Diskussionsbereitschaft und das rege Interesse an dem Fortgang der Arbeit bedanken.

Für die Zweitkorrektur dieser Arbeit danke ich Frau Prof. Dr. Dagmar Meyer zu Heringdorf aus dem Institut für Allgemeine Pharmakologie und Toxikologie der Johann Wolfgang Goethe-Universität Frankfurt am Main.

Bei Dr. habil. Andreas von Knethen möchte ich mich für die Betreuung während der gesamten Zeit meiner Doktorarbeit bedanken.

Ein ganz besonderer Dank gilt Anne-Marie Kuhn und Christian Werno für die langjährige Freundschaft, Begleitung und Unterstützung während des Studiums und der Doktorarbeit.

Weiterhin möchte ich mich bei Carla, Nico, Martina, Heidi, Steffi und Tobias für ihre Unterstützung und das gute Arbeitsklima bedanken.

Bei meiner Familie und meinen Freunden bedanke ich mich für die bedingungslose Unterstützung während meiner gesamten Doktorarbeit. Insbesondere Pedro möchte ich danken für das liebevolle Abschlussgeschenk, an dem wir uns beide ein Leben lang erfreuen werden.

11 Curriculum vitae

Name	Vera Diana Barra
Geburtstag	13.11.1980
Geburtsort	Coimbra, Portugal
Nationalität	luxemburgisch

Promotion

09/2006 bis vsl. 07/2010	Promotion im Fachbereich Medizin, Institut für Biochemie I / Pathobiochemie am Universitätsklinikum Frankfurt am Main

Studienverlauf

07/2006	**Abschluss: Diplom Biologin**
	Abteilung für Zellbiologie, Technische Universität Kaiserslautern
2001 bis 2006	Studium der Biologie an der Technischen Universität Kaiserslautern

Schulische Laufbahn

07/2000	**Abschluss: Allgemeine Hochschulreife**
1993-2000	Gymnasium Lycée de Garçons Esch-sur-Alzette, Luxemburg
1987-1993	Grundschule Consdorf, Rosport, Esch-sur-Alzette, Obercorn, Luxemburg

Vera Diana Barra

Frankfurt am Main, den 19. Juni 2010

12 Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Arbeit mit dem Titel

"Regulation of arginase II expression in macrophages by supernatants of apoptotic cells"

im Institut für Biochemie I – Pathobiochemie unter Betreuung und Anleitung von Prof. Dr. Bernhard Brüne mit Unterstützung durch Dr. habil. Andreas von Knethen ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Teile der vorliegenden Arbeit werden im folgenden Publikationsorgan veröffentlicht:

Barra V, von Knethen A, Weigert A, Brüne B. Apoptotic cell-derived factors induce arginase II expression in murine macrophages by activating ERK5/CREB. **Cellular and Molecular Life Sciences. 2010. [in revision]**

Vera Diana Barra

Frankfurt am Main, den 19. Juni 2010